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IMPACT OF ALCOHOL AND DRUG ABUSE ON HIPPOCAMPAL NEUROGENESIS IN HUMANS

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Stockholm 2018

Cover image: A glimpse of the granular cell layer and the subgranular zone in human hippocampus with a positive staining for doublecortin (DCX) and the neuronal nuclear marker (NeuN), markers for immature and mature neurons respectively.

Published by Karolinska Institutet.
Printed by E-print AB 2018

ISBN 978-91-7831-267-2

Impact of Alcohol and Drug Abuse on Hippocampal Neurogenesis in Humans

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family,

“நோய்நாடி நோய்முதல் நாடி அதுதணிக்கும்

வாய்நாடி வாய்ப்பச் செயல்” – (திருக்குறள் ≃ 5 BC)

“Diagnosing the disease, detecting its root cause,

discerning its cure and then act aptly” – (Thirukkural ≃ 5 BC)

ABSTRACT

Hippocampus is one of the few brain regions in which adult neurogenesis is known to occur. Adult neurogenesis in the hippocampus is considered to be important for higher cognitive function, most notably in memory processes and mood regulation. Alcohol abusers are often diagnosed with memory dysfunction. Several animal studies have reported impairment of alcohol on adult neurogenesis in hippocampus, however in studies of adult human alcohol abusers, no conclusive results have been obtained so far. The work presented in this thesis focuses on understanding the effect of alcohol on hippocampal neurogenesis and is based on studies of carefully phenotyped postmortem human subjects. The studies include the effect of chronic abuse of alcohol on hippocampal granule cells, analyzed using stereological principles and determination of the densities of neural stem/progenitor cells and immature neurons in dentate gyrus. Further, hippocampal cell turnover was studied regarding the effect of alcohol and cocaine using retrospective bomb-pulse derived carbon-14 birth dating procedure.

In Paper I we assessed the effect of alcohol on proliferating cells, stem/progenitor cells and immature neurons using immunohistochemistry with antibodies against Ki67, Sox2 and DCX in the subgranular zone and other layers of the dentate gyrus of the hippocampus. All subjects included in this study had an on-going alcohol abuse for at least four weeks prior to death, a time period that was chosen based on data about the time that elapse from that a neural stem cell after asymmetric division becomes an integrated neuron in the granule cell layer of the dentate gyrus. We found that alcohol negatively affect the proliferating cells, stem/progenitor cells and immature neurons in the hippocampus. The effect was found to be prominent in the subgranular zone and evenly distributed across the distances from the granular cell layer. Alcohol has more pronounced effect on Sox2-IR cells than DCX-IR cells which suggest that the stem/progenitor cells are primarily targeted and that the immature neurons are secondarily affected.

In Paper II we investigated whether the effect of alcohol on neurogenesis might over time affect the number and density of granule cells in the hippocampus. A morphometric method based on the principle of optical fractionator of stereology was applied on blocks of hippocampus at a standardized anatomical location. Neuronal nuclear antigen NeuN was used as a marker for mature granule cell in this study. We found that alcohol significantly reduces the total number and density of granule cells in addition to a decrease in the volume of GCL in hippocampus. We also report that the significant difference in density was primarily due to a reduction of granule cell number. There are substantial inter-individual differences in

granule cell numbers, and alcohol seemingly has a stronger impact on this than age of the subjects.

In Paper III we studied the difference in neuronal and non-neuronal cell turnover rate in control, chronic alcohol abusers and cocaine abusers. We have used a retrospective ^{14}C birth dating procedure to estimate the average age of hippocampal cell populations and mathematical modeling to calculate the turnover rate of hippocampal cells. The turnover rate of both neuronal and non-neuronal cells in cocaine abusers were indistinguishable from control subjects, whereas we observed a lower turnover rate in alcohol abusers compared to controls. However, this difference was not statistically significant when the results were corrected for the age of the subjects. Due to an increased loss of hippocampal neurons in alcoholics, it cannot be excluded that the true turnover rates may be lower in this group.

In conclusion, in this thesis, we have found support for impairment of neurogenesis in the hippocampus in alcoholics and that alcoholics over a lifetime have lost a substantial portion of their granule cells, which may be explained by both a reduced addition of new cells to the dentate gyrus and an increased removal of cells. Using ^{14}C analysis of neuronal nuclei we could not detect a significant difference in turnover of granule cells between alcoholics and controls. A mathematical modeling considering the effect of cell loss in alcoholics and/or a separate analysis of ^{14}C in the granule cell population would be desirable to more in detail understand the dynamics.

LIST OF SCIENTIFIC PAPERS

I. **Effects of Alcohol Abuse on Proliferating Cells, Stem/Progenitor Cells, and Immature Neurons in the Adult Human Hippocampus**
Tara Wardi Le Maître, **Gopalakrishnan Dhanabalan**, Nenad Bogdanovic, Kanar Alkass and Henrik Druid
Neuropsychopharmacology, 2018; 43(4): 690-699.

II. **Hippocampal granule cell loss in human chronic alcohol abusers**
Gopalakrishnan Dhanabalan, Tara Wardi Le Maître, Nenad Bogdanovic, Kanar Alkass, Henrik Druid
Neurobiology of Disease, 2018; 120: 63-75.

III. **Effect of Alcohol and Cocaine Abuse on Neuronal and Non-Neuronal Cell Turnover in The Adult Human Hippocampus Using ^{14}C-birth dating Procedure**
Kanar Alkass, Tara Wardi Le Maître, **Gopalakrishnan Dhanabalan**, Samuel Bernard, Embla Steiner, Kirsty L. Spalding, Jonas Frisén, Deborah C. Mash, Henrik Druid
Manuscript

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LIST OF ABBREVIATIONS

^{14}C	Carbon-14 isotope
AMS	Accelerator mass spectrometry
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CA	Cornu Ammonis
CAST	Computer assisted stereological tool
DAB	3,3'-diaminobenzidine
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
GABA	Glial fibrillary acidic protein
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidase
IHC	Immunohistochemistry
LTP	Long term potentiation
ML	Molecular layer
MRI	Magnetic resonance imaging
NMDA	N-Methyl-D-Aspartic acid
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PSA-NCAM	Polysialylated-neural cell adhesion molecule
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SGZ	Subgranular zone
Sox2	Sex determining region y-box 2

INTRODUCTION

Hippocampus, an anatomical brain region located in the medial temporal lobe has important function in learning, memory and in mood regulation. It is one of the few brain regions in which adult neurogenesis is considered to occur throughout the life time of humans with modest decline with age. Experimental studies on animals suggest that the formation of new neurons is crucial for hippocampal functions, to adapt to new situations. Impairment of neurogenesis has been observed in alcohol and drug abuse in animal studies[1,2]. In this thesis, the effect of alcohol on proliferation, numbers of stem/progenitor cells and immature neurons in the dentate gyrus is studied in human subjects with an on-going alcohol overconsumption and in control subjects. The impact of long-term alcohol abuse on total number of granule cells has also been studied. Finally, the turnover of hippocampal cells in chronic alcohol and cocaine addicts is also investigated using bomb-pulse derived radiocarbon birth dating. High alcohol intake for longer period is associated with general brain atrophy including atrophy of hippocampus. The main hypothesis of this thesis is that alcohol and drugs of abuse might have an impact on the formation of new neurons in hippocampus, which may represent one of the morphological substrates of memory impairment in alcohol/drug addiction. Previous postmortem studies on human alcoholics have failed to document a decrease in number of neurons, which may due to inclusion of small cohorts and less well-defined inclusion criteria for alcoholics. In this thesis the effects of both short and long-term alcohol and cocaine abuse on neurogenesis, and the total number and turnover of neurons in the hippocampus, are studied.

ALCOHOL ABUSE

Socio-economic impact of alcohol

Alcohol abuse is associated with social-economic impact in addition to the medical consequences that contribute to the severity of the disease worldwide. According to the World Health Organization (WHO), excessive alcohol use is the third leading cause of disease and disabilities in the world[3]. Alcohol affects the human body in many ways; in particular excessive use alcohol may lead to liver cirrhosis, and increase the risk for cancer and cardiovascular diseases[3,4]. Chronic alcohol abuse is one of the most prevalent psychiatric disorders in the western world, and also one of the comorbid disorders accompanying other mental disorders such as depression and anxiety[5]. Further, alcohol abuse and addiction to alcohol cause enormous economic cost to the society; in the US the annual cost is estimated to $250 billion[6] and in Sweden the annual cost related to alcohol abuse is estimated to 100

billion SEK[7,8]. Apart from all these medical and economic consequences, alcohol exerts a heavy burden to the family, friends and social life of the affected individual[9].

Physiological effects of alcohol

Alcohol has wide physiological effects on brain and other parts of the body. In humans, alcohol acts as a central nervous system depressant with mild stimulating effects on some parts of the brain[10]. At higher levels, alcohol may cause disorientation, coma and can even lead to death. Alcohol is metabolized in the liver, which breaks down alcohol to acetaldehyde and then further to acetic acid by alcohol dehydrogenase and aldehyde dehydrogenase, respectively[11]. Alcohol, when consumed in large quantity over long periods of time may lead to fatty degeneration and even fibrosis (cirrhosis) of the liver due to increased fatty acid synthesis, inhibition of their oxidation, and lactate-stimulated collagen formation, all basically in turn due to the formation of the reduced form of NADH and the metabolite acetaldehyde during ethanol metabolism by alcohol dehydrogenase[12]. Chronic alcohol associated liver cirrhosis is characterized by large fatty droplets in the liver epithelial cells, sometimes also containing alcohol hyaline inclusions, portal lymphocyte infiltrates, proliferation of the bile ducts and scarring, which eventually produces pseudolobules[12,13]. Thus, this form of macro- and microscopic visible alcohol-related liver damage constitutes one very specific evidence of long-term alcohol overconsumption. Alcohol is known to cross the blood brain barrier, a barrier that protects the brain from the entry of undesired molecules, but which also regulates the elimination of waste molecules in the brain[14,15]. Alcohol at high concentrations in the blood acts as vasoconstrictor by increasing blood pressure that can leads to stroke[16,17] and functional brain disorders like vascular dementia[18,19]. Even moderate doses of alcohol for longer period of time can lead to vitamin deficiency as in Korsakoff's syndrome, resulting in memory loss, emotional disturbance and ataxia[20,21].

Misuse, abuse and addiction

In postmortem human studies on alcohol and substance abuse, the terms misuse, abuse and addiction are often used to categorize studied subjects since ICD or DSM codes generated from medical records case management systems are typically not available. This is compensated by the retrieval of information on the use of alcohol and/or drugs from other sources that are often not used, or available for health care professionals. For the present studies, the source of information is not critical, rather the reliability of the information. Even if medical records are often considered the gold standard, for the formation of groups of addiction used in this thesis a synthesis of data from several sources is used, since the aim is

to explore association between overconsumption of alcohol or cocaine and hippocampal neurogenesis rather than the association between addiction and neurogenesis in hippocampus, hence regardless if the subjects are addicted to alcohol or certain other drugs or not.

According to the World Health Organization (WHO), misuse is a use of substance for a purpose not consistent with legal or medical guidelines, i.e. nonmedical use of prescription medications[22]. Abuse is defined as persistent or sporadic excessive use of a psychoactive substance despite knowledge of having a persistent or recurrent social, occupational, psychological or physical problem that is caused or exacerbated by the use of the substance[22]. Addiction to a substance is defined as repeated use of a psychoactive substance or substances to the extent that the user (referred to as an addict) is periodically or chronically intoxicated, shows a compulsion to take the preferred substance (or substances), has great difficulty in voluntarily ceasing or modifying substance use, and exhibits determination to obtain psychoactive substances by almost any means[22]. In this study, the cases identified as alcoholics and cocaine subjects are abusers for those substances. So, the excess consumption for a longer period was used to include them in the study whether or not they also fulfilled the criteria for addicts. Having said that, most of the Swedish subjects were actually also diagnosed with alcohol addiction.

Effect of alcohol on hippocampus and other brain regions

Alcohol affect different brain regions and long-term excessive alcohol consumption results in general and regional brain atrophy, although it is not quite clear whether this also requires that the subject suffer from vitamin B_1 deficiency[23-25]. Pathological findings in animals show that both grey and white matter volume reductions contribute to the alcohol induced brain atrophy[26-29]. Neuronal loss has been shown in specific regions of cerebral cortex, hypothalamus and cerebellum in alcoholics[30-32]. Evidence of lesions and volume shrinkage has been found in the mammillary bodies of alcoholic subjects with Wernicke-Korsakoff syndrome[33]. In experimental animal studies, there is a reduction in hippocampus volume in alcohol abuse models[34-36]. Recent studies on humans have also reported a reduction in hippocampus volume in association with chronic alcohol abuse[37-39] and this reduction in hippocampal volume in alcoholics in humans is also observed in meta-analysis on human findings[40].

The main purpose of this thesis is to elucidate the short-term and chronic effect of alcohol and drugs on adult neurogenesis in hippocampus, particularly the effect of alcohol on proliferation, stem cell pool, and numbers of immature neurons. The studies also aim at finding out whether alcohol or cocaine affects the turnover dynamics of neuronal and non-

neuronal cell populations in the hippocampus. To this end, different cohorts of deceased donors selected for the particular study purpose, and different methodological approaches have been used.

Since the main focus of this study is hippocampus, it is important to know its basic anatomy; its types of cells, and their neural connections with other brain regions; its function in adult brain; and its pathology in diseases. Hence, these topics are discussed in the following sections.

HIPPOCAMPUS

This section focuses on hippocampus anatomy with the basic two laminar structures, the inputs and outputs to the dentate gyrus and the function of hippocampus in adult brain.

Basic Anatomy

Hippocampus, localized in the temporal lobe of the brain, was described by the anatomist Julius Caesar Arantius in 1579, whom because of its appearance called it hippocampus, Latin for sea horse. Hippocampus has a laminar structure, i.e. all principal cells are arranged in separate subdivisions. The hippocampal formation comprises the dentate gyrus (DG) and the hippocampus proper with four subdivisions: CA-Cornu Ammonis (CA1, CA2, CA3 and CA4), subiculum, presubiculum and parasubiculum. Entorhinal cortex (EC) is often included in the basic anatomy of hippocampus formation, since it is from there afferents and efferent of hippocampus is relayed to neocortex. A coronal section that shows the basic anatomy of the human hippocampus formation can be seen in Figure 1.

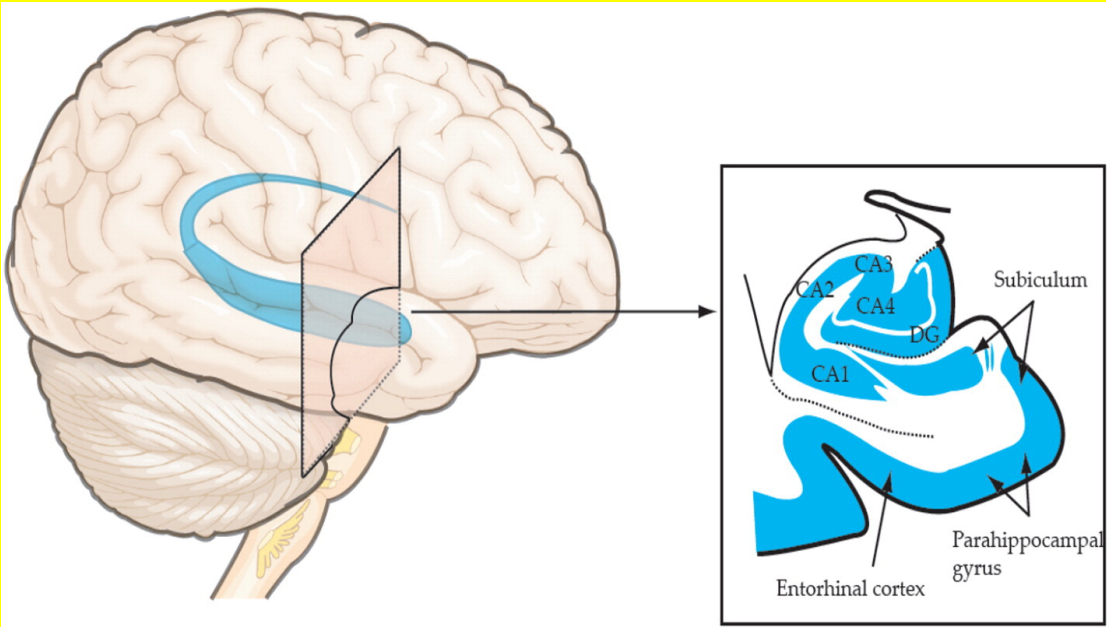

Figure 1: Basic anatomy of the human hippocampal formation from a coronal section is shown. Reprinted from Kang et al., 2015 with permission.

Cornu Ammonis (CA)

Cornu Ammonis or Hippocampus proper is divided in to six layers: alveus, stratum (st.) oriens, st. pyramidale, st radiatum, st lacunocum and st moleculare. The main elements of CA are the pyramidal neurons in st. pyramidale. Pyramidal neurons project mainly via alveus to the septal nucleus, to other pyramidal neurons via associative fibers and cross the contralateral hippocampus. There are four subfields which Lorente de No in 1934 named CA1-CA4. The CA1 sub-field consists chiefly of pyramidal cells that are scattered in the large pyramidal stratum in human hippocampus, but which are small and narrow in rats[41]. CA2 is composed of large and densely packed cells in contrast to CA1[42]. CA3 consists of less densely packed pyramidal cells but less pronounced. Moreover, CA3 has a specific subregion, stratum lucidum layer, which consists of mossy fibers, the projection axons from dentate gyrus. CA4 is situated within the cavity of DG known as hilus and consists of large, ovoid and scattered cells surrounded by mossy fibers. The CA1 is considered as the main output of the hippocampus projecting to the fimbria and sending collaterals to the subiculum. The subiculum has three layers that are similar to the CA region: the pyramidal layer, molecular layer and polymorph layer. The CA1 pyramidal cell axons project to the pyramidal and molecular layer of subiculum, in which the former in turn projects to the deep layers of the entorhinal cortex[43]. Thus, the entorhinal cortex acts as a relay station further transferring processed information from the hippocampal formation into the cortex and some other brain regions[44].

Dentate Gyrus (DG)

The current study is mainly focused on the DG, a well-decanted structure that surrounds the CA4 field of CA. It consists of three layers that are easily visualized on the coronal sections of the hippocampus: the granular cell layer (GCL), the sub-granular zone (SGZ) and the molecular layer (ML). These three layers together form fascia dentata and the laminar pattern observed is conserved among mammals[45-47]. The granular cell layer consists of somata of densely packed granule cells making this layer easily identified on histology slides. Their axons (often referred as mossy fibers) are non-myelinated and run through the polymorphic layers to CA4 and CA3. The granule cells have dendrites that protrude into the ML[48]. The SGZ contains precursor stem cells that are polymorphic and this layer runs parallel to the GCL between CA and GCL. The width of the SGZ in the human brain is not well characterized and may be several hundred microns, although most researchers believe that the highest activity is in the areas closest to GCL, but since the borders of the human SGZ has not as yet been defined, histological studies of SGZ have been performed on areas of variable width[49,50]. The molecular layer of DG consists mainly of inhibitory neurons and of dendrites of granule neurons and afferent axons.

Circuits of the Dentate Gyrus – inputs and outputs

The dentate gyrus is the main entry point of the hippocampal trisynaptic network. The tri-synaptic perforant pathway from entorhinal cortex innervate the ML that enter into DG[45]. The lamellar set of unidirectional connections are as follows: starting from axons of entorhinal perforant pathway to the dendrites of granule cells in the molecular layer, the axons of dentate gyrus known as mossy fibers transverse the polymorphic layer to CA4 and CA3; the axon collaterals of CA3 known as Schaffer collaterals projects to CA1 laterally and from CA1 the axons projects back to entorhinal cortex[51,52]. However, the trisynaptic pathway is not entirely linear as explained and there are additional direct inputs of perforant pathway as well into CA3 region. The inputs from the cortex is essential in the formation of episodic memories that enters through this pathway into the dentate gyrus via entorhinal cortex.

Information arriving from the isocortical areas is forwarded towards entorhinal area and hippocampus implying that new information has to be processed via entorhinal cortex and hippocampus before final storage in the neocortex. Hippocampus has specific intrahippocampal circuits: polysynaptic and direct pathways. The origin of polysynaptic pathways are neurons in layer 2 of EC which has been named perforant pathways since they pass through subiculum on the way to ML. Dentate granule cells extend mossy fiber to CA3 and CA4 neurons which are interconnected with the subiculum and with CA1 pyramidal neurons via Schaffer collaterals. Finally, projection neurons in CA1 are sending axons to the

subiculum, septal area and to the deep layers of EC. Deeper layer III in EC is sending direct projection to CA1.

The dentate gyrus has many types of resident GABAergic interneurons in the molecular and polymorphic layer exerting some inhibitory effect on granule cells and pyramidal cells. The GABAergic interneurons in the hippocampus have influence on hippocampal function[51,53]. The dentate gyrus also receives external regulatory connections outside the hippocampus: cholinergic fibers from septal nuclei[54], commissural fibers that connects the two hippocampus via fornix[55], serotonergic fibers from raphe nuclei[56], dopaminergic fibers from ventral tegmental area[57]. Hippocampus also has direct connection with the associative neocortex[58].

Function of hippocampus in the adult brain

The human hippocampus has been known to play a critical role in memory since the seminal finding by Scoville and Milner[59] reported on patient HM. After performing a lobectomy of the temporal lobes as a treatment to intractable epilepsy, HM was diagnosed with severe and permanent amnesia while his general mental abilities remained intact. Since HM's lesions were substantial it was difficult to identify the importance of specific subregions in the temporal lobe[60]. The authoritative proof came 50 years after the patient HM study, when functional deficits of patients with more selective hippocampal damage were reported[61-63].

Today it is well known that hippocampus has important function in learning and memory and in the regulation of mood[64]. Learning and memory functions of hippocampus are dependent on activity related adaptive changes at synaptic and network levels and this can be modified by experience[65]. The adaptive changes are referred to as hippocampal plasticity which is mainly due to neurogenesis in the SGZ of hippocampus that will be discussed in the subsequent sections.

Long-term memory is comprised of the declarative memory and non-declarative (procedural) memory system. Hippocampus has an important function in declarative memory that includes both episodic and semantic memory[66]. The declarative memory system encompasses memories for facts and events mediated by the hippocampus in the medial temporal lobe of the human brain. The persistent craving for alcohol and drugs even after long-term withdrawal implicates the presence of drug related memory in the hippocampus that may be due to reduction of functional replacement by adult generated neurons.

Comparison of hippocampus anatomy and function: rodents vs primates and humans

This section aims at describing the hippocampal anatomy and function in different species to provide an understanding of the results obtained from different studies. The hippocampus longitudinal axis is defined as ventral-dorsal in rodents and anterior-posterior in primates and humans on the basis of long axis of hippocampal orientation. The long axis of the hippocampus in the rat must undergo an orthogonal rotation in order to have same orientation as in primates and humans. The entorhinal cortex is the interface between the hippocampus proper and neocortex through which both input and output pathways run in addition to direct connections in all the three species[67]. The anterior part of the hippocampus is connected to the entorhinal cortex in humans and primates while in rodents with the dorsal (=posterior) part of the hippocampus. In humans, the posterior part of the hippocampus is associated with memory function which is dorsal part in rodents[68]. As previously described, hippocampus receives input from the entorhinal cortex and intrahippocampal pathways. However, hippocampus have also direct and polysynaptic pathways to and from different cortex regions. Hippocampus receive direct inputs from inferior temporal association cortex (connected to inferior visual system) through perirhinal cortex and sends direct output to deep layers of entorhinal cortex and prefrontal cortex[51]. The prefrontal cortex has a well-known role in decision making, which is poorly developed in rodents[69]. The polysynaptic output from hippocampus reaches the mamillary bodies and thalamus via fornix, which in turn have projections to frontal, anterior and posterior cingulate cortices. The posterior parietal association cortex have polysynaptic input to hippocampus via parahippocampal gyrus and entorhinal cortex[51]. Hippocampus is also known to receive emotional responses from prefrontal cortex and send projections to nucleus accumbens and amygdala[70,71]. The above description on hippocampal connections with other brain regions and function aims at helping the reader to give a basic understanding of the differences in hippocampal connection between species.

HIPPOCAMPUS PATHOLOGY AND FUNCTION DEFICITS IN ALCOHOLISM AND DISEASES

Hippocampal atrophy and diseases

Imaging studies on the human brain and hippocampus in the last decades have been linking the hippocampal atrophy, i.e. the decrease in the volume of hippocampus, to learning and memory related dysfunctions. In healthy adults the size of the hippocampus is positively correlated with hippocampus dependent memory functions such as declarative and episodic memory functions[72,73]. Several neurologic diseases such as Alzheimer's disease, dementia

with Lewy bodies, as well as traumatic brain injury have negative effects on the hippocampal volume and memory performances as assessed with a variety of tests [72,74-76].

Hippocampus is also involved in modulation of mood and the duration of depression has been shown to correlate with the severity of hippocampal atrophy. Several evidences suggest that the hippocampal atrophy due to long-term depression persists even after remission from depression[77,78] and treatment with tricyclic anti-depressants has shown larger DG and GCL volume in the hippocampus[79].

Hippocampal function and pathology in alcohol abuse

Hippocampus is one of the key brain regions found to be most sensitive to the effects of alcohol. The well-known functions of hippocampus such as semantic and spatial memory are particularly vulnerable to the effects of alcohol[80,81]. A general impairment in the structure and function of hippocampus was seen after chronic alcohol exposure in experimental models[80,82,83] and in human brain studies[84-86]. The regional brain damage observed in chronic alcoholics is often found associated with hippocampal damage and related memory impairment in humans[81,83]. This is in turn supported by experimental alcohol addiction models in which a significant impairment in working memory deficits has been observed[87,88]. These studies suggest that the effect of alcohol on cognitive function is mainly through its effects on hippocampus[89]. The structural and functional changes in the hippocampus induced by chronic alcohol abuse have been investigated in several studies and the reported effects include neuronal loss[90,91], glial cells loss[92], dendritic alteration in hippocampal neurons[93,94], reduction of hippocampal neurons[1], and decrease in long term potentiation (LTP)[95].

Alcohol induced neurotoxicity in hippocampus and the brain cytotoxicity

The major neurotoxic effect of alcohol in the brain is considered to be due to oxidative stress, which may be related to effects on NMDA (N-methyl-D-aspartate) receptors and associated activity through GABA (gamma-aminobutyric acid) receptors, whether or not this is considered to be caused by excitotoxicity. The NMDA receptor is also proposed to have major role in different phenotypes of alcoholism such as tolerance, dependence, withdrawal, craving and relapse[96,97]. Alcohol induced neurodegeneration through excitotoxicity is mostly shown in *in vitro* studies and a very few *in vivo* studies that claims neurodegeneration through NMDA receptors has not shown enough quantitative effect due to NMDA receptors[98]. Both NMDA and GABA receptors are present in the dentate gyrus and CA regions[99], but most of the authoritative proof of expression of these receptors either on stem/progenitor cells or granule neurons in the hippocampus is from electrophysiological studies[100-102]. The inhibitory

action of ethanol on the activity of NMDA receptor was demonstrated using electrophysiological studies on hippocampus[103] and also amygdala[104] and striatum[105,106]. Chronic ethanol exposure has been suggested to induce excitotoxicity through hypersensitivity of NMDA receptors[107-109]. Crews et al. have suggested that NMDA receptor mediated oxidative stress resulting in nitric oxide formation in the brain is a typical effect of high dose alcohol exposure[36]. Brain cytotoxicity could also occur through increase in the level of proinflammatory cytokines due to oxidative stress induced by alcohol[110]. Nevertheless, excitotoxicity and oxidative stress is suggested in alcohol related brain damage, it is not well studied in humans as several factors such as drinking pattern and other factors over the years should be extracted carefully in order to quantify neurodegeneration over the lifetime.

NEUROGENESIS

Neurogenesis is the process by which self-renewing, pluripotent, neural stem cells (NSC) become neurons. Neurogenesis is known to be prevalent during pre-natal development and it is a vital process for populating the developing brain. Neurons are the fundamental unit of the nervous system through which information is being processed and transferred to different parts of the brain. Whereas embryonic neurogenesis is critical in the initial development of the brain, adult neurogenesis is responsible for the continuous development and integration of new memories in the brain.

Adult Neurogenesis

With the notable exception of the granule cells in the dentate gyrus and olfactory bulb, most neurons in the primate brain including humans, appear to be generated before birth or during the early neonatal period and then persist during many decades of the primate life span[111]. Adult neurogenesis is a process by which generation of new neurons from the pluripotent, self-renewing neural stem cells integrate into existing circuits after the postnatal development stages. In mammals, adult neurogenesis occurs in two principle regions – the subgranular zone of the DG and the subventricular zone of the lateral wall of the lateral ventricles.

The earliest evidence of adult neurogenesis was reported in 1912 by Ezra Allen, when Allen showed mitotic figures in the lateral ventricles of albino rats[112]. Despite this observation, the adult brain was strongly believed to be incapable to produce new neurons during those times. It was not until Altman and Das in their seminal study 1965, that this dogma was questioned.

They demonstrated that adult hippocampus is the host to birth and death of new cells for the first time in adult rat dentate gyrus[113] using auto-radiographic labeling technique. This study was replicated by Kaplan more than a decade later using electron microscopy showing that the adult born cells were indeed neurons[114]. Fernando Nottebohm in 1983, demonstrated the functional integration of adult generated neurons for the first time in the song system of adult birds, supporting the seasonal learning in canary birds[115]. Almost more than a decade after Nottebohm´s report, a number of researchers showed adult neurogenesis in hippocampus of rodents and different species of primates using BrdU labeling and immunohistochemistry (IHC)[116-118]. Hence, adult neurogenesis started to become more widely accepted as an integral part of brain plasticity in the late 1990s.

In humans the first direct evidence for adult neurogenesis was provided by Eriksson et al., 1998 observing that cells in the adult brain undergo cell division and eventually matured into new neurons in the dentate gyrus of hippocampus[50]. A recent seminal study by Spalding et al., using a novel ^{14}C dating procedure reported that in adult humans approximately 700 new neurons are added per day in each hippocampus, corresponding to an annual turnover rate of 1.75% of neurons within the renewing fraction, with a modest decline during aging[119]. Hippocampus is the brain structure important for learning and memory formation and it has been proposed that the integration of new neurons is essential for structural and functional integrity of hippocampus[120]. Hence, if it is accepted that neurogenesis continues to go on in the adult human brain, the production of new neurons most likely follows the same principles as observed in other species. From animal studies the following sequences have been established. In the dentate gyrus, a hippocampal subregion, a homeostatic pool of young neurons is maintained to allow for neurogenesis. New granule cells are consistently generated in the subgranular zone (SGZ) of the DG; these immature neuronal cells migrate to the granular cell layer (GCL), and extend their axons and dendrites to their target areas[121].

These newborn neurons in the hippocampus have distinct electrophysiological properties, as they need only weaker stimulation to get activated (hyper-excitable)[122]. As hippocampus is known for its role in learning new things that are associated with weaker stimulation, the hyper-excitability of newborn neurons support the formation of new memory and makes it distinct from existing memories[123]. Animal studies have shown that newly formed neurons in the adult DG make distinct contributions to learning and memory[124,125].

In animals, the newly produced neuroblasts in the SVZ migrate via the rostral migratory stream to the olfactory bulb, where they differentiate and replace interneurons undergoing rapid turnover[126]. The same is true for hippocampus but since the neuronal stem cells are located mainly in the SGZ, they do not have to travel far to reach their destination in the GCL. It has been estimated that 9000 new cells are produced each day in rat dentate gyrus of which 50% express neuron specific markers. This rate in cell proliferation equals nearly 6% of the mature granule cell population newly formed every month[127]. However, in olfactory

bulb due to higher replacement of existing neurons, the neurogenesis is essential to maintain the number of neurons[128]. Also the functional role of newborn neurons in the olfactory bulb is poorly understood, in spite of the proposed role in odor discrimination[129]. In contrast to the olfactory bulb, the dentate gyrus seems to show very low neuronal death rate[128]. The recent debate on the existence of neurogenesis have re-ignited the topic and it concludes that studies on adult hippocampal neurogenesis not only focuses on its existence but also on the possible functional implications[130-132].

Stages of adult neurogenesis in the DG

Adult neurogenesis in the DG is observed constitutively throughout the adult life and various physiological and pathophysiological conditions are known to affect adult neurogenesis in DG. In addition to this, environment and behavior also influence adult neurogenesis in the DG[120,133-136]. Adult neurogenesis in hippocampus is phenotyped into different cell types on the basis of expression of different molecular markers that defines the stages involved in the process of adult neurogenesis. Since adult neurogenesis is a continuous process all types of developmental markers can be found at any given time (Figure 2).

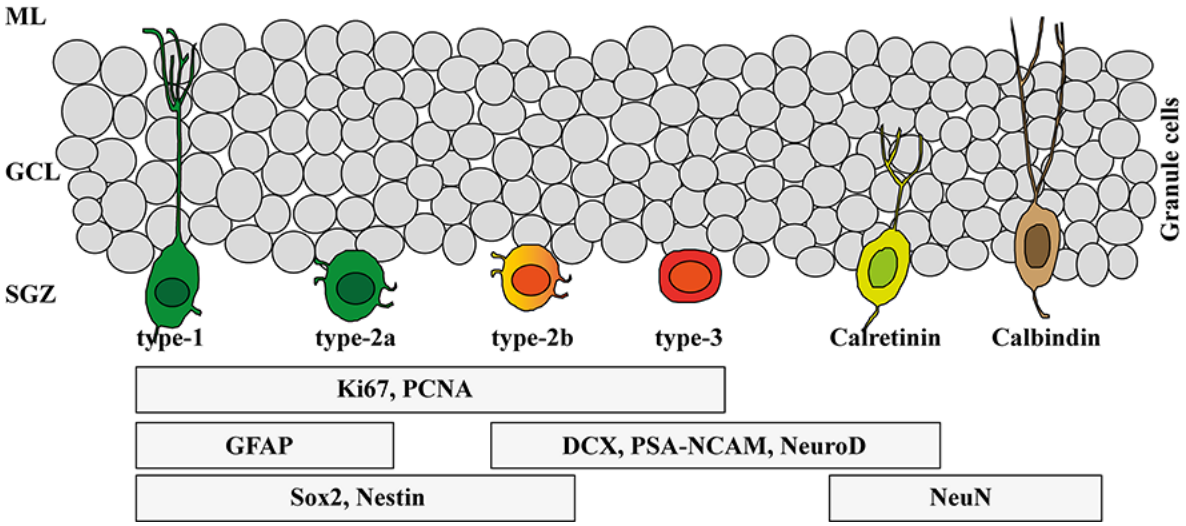

Figure 2: Cell types in the stages of adult neurogenesis in the human hippocampus has been shown with morphology and molecular markers from different human and animal studies. The scope of all possible co-localizations has not been studied in this thesis.

The first stage (type-1 cells) in the adult neurogenesis is the proliferation stage. Newly formed cells in this phase express glial fibrillary acidic protein (GFAP), Sox2 and nestin[137-139]. In the SGZ these cells represent putative stem/progenitor cells although it is known that a subset of astrocytes also express GFAP and nestin that can give rise to new granule cells[140]. The cells in this phase are proliferative in nature and express markers for proliferation such as Ki67 and PCNA[141]. The type-1 cells show radial glia like morphology and may either undergo a symmetric cell division that maintains the stem cell pool in the SGZ or undergo an asymmetric cell division and give rise to progenitor cells[142].

The second stage is the type-2a and type-2b cells that are transit-amplifying cells that differentiate into immature neurons in the SGZ. The type 2a cells are proliferative, nestin positive and GFAP negative[141,143]. It is in this phase of neurogenesis a stem cell is thought to commit to neuronal lineage. In the type-2b stage, cells start expressing doublecortin (DCX) and the polysialylated neural cell adhesion molecule (PSA-NCAM) but stop expressing nestin[121,138,143].

The cells that are completely negative for nestin and express PSA-NCAM and DCX are referred as type-3 cells[121,141]. These cells migrate a short distance from SGZ into the granule cell layer (GCL) of the DG.

In stage 4, the immature neurons extend their dendrites towards the molecular layer of DG and send their axons towards the CA3 pyramidal layer into the hilus. It is in this stage the newly formed neuronal cell becomes post mitotic. The neuronal cells in this stage still express DCX and PSA-NCAM[141,142,144]. Also, these early post mitotic neurons both in humans and mice transiently express the calcium binding protein calretinin[141,145,146] and the neuron specific nuclear protein NeuN[119,145,147].

In the final stage, the matured granule neurons establish their afferent and efferent synaptic contacts for receiving inputs and sending outputs from entorhinal cortex and hilar CA3 region, respectively. However, it is known that the trisynaptic hippocampal circuit is not absolutely linear. In this maturation stage the calretinin seems to be exchanged for calbindin[141,142,145]. Thus the mature granule cells in the DG express calbindin[148,149] and become functionally integrated in the hippocampus network[150]. Functionally matured granule cells in DG express NeuN in both humans and animals, and this marker is therefore extensively used for the identification of mature granule cells in several seminal studies[119,141,151,152].

The entire maturation process of adult neurogenesis is estimated to be 6 weeks in adult non-human primates[153] and it is assumed that the time frame is similar in the human hippocampus.

STUDYING ADULT NEUROGENESIS AND CELL TURNOVER IN HIPPOCAMPUS

In this section, the approaches to study adult neurogenesis used in this thesis; immunohistochemistry, stereology, and bomb-pulse derived ^{14}C birth dating procedure, are briefly explained.

Histological markers of adult neurogenesis

There are many markers that have been used to identify neurogenesis. BrdU immunohistochemistry is used to identify newly generated cells in S-phase in the brain, a method that was developed by Miller and Nowakowski in 1988[154]. Since BrdU immune labeling will stain all cells in S-phase, in general the identification of BrdU in the adult brain indicates cell genesis rather neurogenesis specifically. Hence BrdU labeling of cells can be used to identify neural or glial cells only if additional immunohistological markers are used in combination. Eriksson et al., in 1998, performed the only study of adult neurogenesis in humans using BrdU and identified newly formed cells in the adult hippocampus[50]. Since it is not possible to use BrdU in humans today because of patient safety concerns, studies on adult hippocampal neurogenesis in humans have to instead rely on the use of immunological markers of different cell types representing different stages of neurogenesis[141,155].

Ki67 is one of the most used markers to study proliferation in adult human and it is also extensively used in several animal studies. It is a nuclear protein expressed in all phases of cell cycle except resting phase and predominantly in S-phase on all mammalian species from rodents to humans[156]. From animal studies it is known that Ki67 and BrdU expression has a similar pattern in the DG[157]. Ki67 is considered as the most appropriate mitotic marker of proliferation in the studies on adult neurogenesis in humans, since treatment with thymidine analogs are not possible to carry out as in animals.

Sox2 is a transcription factor expressed in neural stem/progenitor cells and it is also a marker for development in both embryonic and adult neurogenesis stages. Sox2 tagged EGFP (Enhanced Green Fluorescent Protein) constructs were identified in the neurogenic regions of sub-ventricular zone and hippocampus[158]. Sox2 expression was observed in proliferating precursor cells and also expressed in adult brain derived stem/progenitor cells[159]. Sox2 is expressed by neural progenitors and astroglia in the adult rat brain[160]. It controls the proliferation and maintenance of the undifferentiated neural stem cell state[161]. Sox2 is one of several suitable markers to study stem/progenitor cells in the DG of human hippocampus.

DCX is a microtubule-associated protein that stabilizes the microtubule assembly and formation of mature microtubule appropriate for migrating neurons[162,163], however the exact function in the adult hippocampus is not yet known. In addition to its role in the migration of immature neurons, it might have important role in neurite development[164]. In the adult human DG, DCX is a marker for immature neurons and the expression of DCX has been observed across the entire lifespan in the DG of human hippocampus[141]. In the adult DG, DCX has been used to analyze dendritic growth of newly generated neurons[165] in addition to provide estimates of the absolute immature neuron numbers[141,155].

GFAP is widely used for histological identification of mature astrocytes in the adult brain. GFAP positive cells observed in the adult neurogenic niche represent cells that are generated during gliogenesis. In the sub-ventricular zone of adult mammals, cells with astrocytic properties give rise to new neurons during adult neurogenesis[166]. Studies on mice show GFAP expression of dividing cells of glial cell lineage in the adult hippocampus[167]. However, in adult humans, GFAP is found to co-express with neuronal lineage markers such as nestin (neuronal stem cell marker) and DCX in the hippocampus[141]. So GFAP-IR in the adult human hippocampus seems to represent cells of glial lineage and also neural stem cells.

PSA-NCAM is a marker for immature neurons in the adult brain and the immunoreactivity is also associated with neural progenitor cells[168]. In addition, it is expressed by type-3 progenitor cells in humans[141]. PSA-NCAM expressing cells in the hippocampus were observed positive for mature neuronal markers (NeuN and Neuro D) and also with DCX[141,169]. The upregulation of PSA-NCAM was observed in association with hippocampus dependent learning tasks [170] and also seems to play an important role in synaptogenesis[171]. Thus, changes in immunoreactivity of PSA-NCAM might not correlate with neurogenesis per se, but can be used as a marker for immature neurons as it parallels the expression of DCX in the hippocampus.

The progenitor cells located in the SGZ of DG proliferate, differentiate and give rise to mature granule cells. The different immunological markers discussed are expressed during different stages of adult neurogenesis, however the up- and down- regulation of markers are not confined to adult neurogenesis stages. There seems to be an overlap in expression patterns of the markers between various of the neurogenesis stages. Hence the use of several markers, studies of co-expressions and of the associated morphology should be considered in when evaluating adult hippocampal neurogenesis, especially in humans.

Stereological studies

Stereology and stereological principles (SP) in morphometry is used to provide an objective estimate of cell numbers in a biological sample (tissue) in many human and animal studies. In order to estimate cell number or density both optical disector and fractionator can be used depend on the thickness of the tissue used. In our study (Paper II) a morphometric method based on optical fractionator method of stereology was used. This method has basic stereological principles of unbiasness: a three-dimensional probe, optical disector and the fractionator, to sample granule cells in the GCL. Since granule cells are large in numbers that are uniformly distributed and have mature neuronal marker NeuN, SP is the right procedure to access the effect of alcohol on granule cell number, density and volume of GCL. The experimental and analytical procedure is explained more in detail in the experimental procedure part of this thesis under morphometric quantification. Stereology has been used to estimate neuron and glial cell numbers in different brain regions including hippocampus in both human and animal studies[172,173]. The difference in total number of neuron and glial cells has also been estimated using stereology in different neurodegenerative diseases[174,175]. In a recent study, stereology has also been used to access the effect of aging on hippocampal neurogenesis in humans[130].

^{14}C birth dating of cells in hippocampus

The concentration of the carbon isotope ^{14}C has been used for dating of biological archeological material since it displays a radioactive decay, which results in a gradual reduction of this isotope after the death of an organism[176]. However, the decay is extremely slow with a halftime of 5730 years, which means that ^{14}C concentrations in modern material is not significantly affected by this decay. The isotope is normally produced in the atmosphere by the interaction of cosmic rays and nitrogen. The ^{14}C produced by this natural process is very low and the production is counterbalanced by a constant emission back to space. Therefore, the ^{14}C levels in the atmosphere and biosphere have remained constant for thousands of years. However, above-ground detonations of nuclear weapons during the cold war between 1955 and 1963 resulted in a dramatic increase in ^{14}C levels in the atmosphere[177,178]. After the test bomb ban treaty in 1963, the levels have decreased exponentially by gradual consumption by the biosphere and from there, diffusion into the ocean reservoirs and finally by emission to space. Regardless of the proportion in the atmosphere, ^{14}C is treated in the atmosphere and the biosphere in the same way as ^{12}C. Hence, ^{14}C in the atmosphere interacts with oxygen to produce carbon dioxide that enters the biosphere through the process of photosynthesis. The process of ^{14}C in the carbon dioxide entry into the biosphere is very fast, which is proven by carbon dating of tree rings, with an average delay of less than 6 months[179,180]. Living organisms exchange carbon from the

atmosphere into their system via metabolism and the stable incorporation of ^{14}C depends on the stability of the biological molecules in the living system (Figure 3). DNA is a very stable molecule in the living system and the synthesis of new carbon backbone occurs only during the replication cycle during cell division, which produces a biological age mark of the cell. In these studies, we take advantage of measuring the amount of ^{14}C in the DNA of the cell in measuring the age of a cell population in the hippocampus.

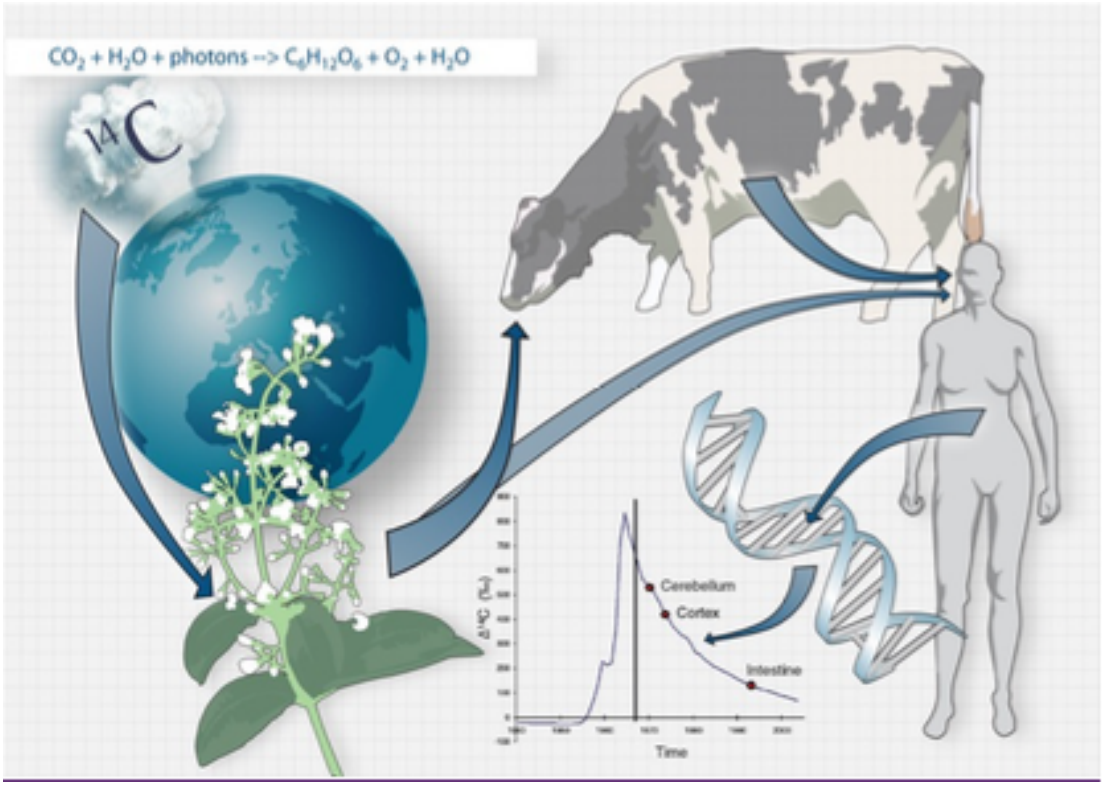

Figure 3: Schematic illustration of bomb-pulse derived ^{14}C from the atmosphere integration into living system and retrospective birth dating of cells. Above-ground nuclear bomb tests during the cold war injected a pulse of ^{14}C into the atmosphere. The ^{14}C reacts with oxygen to form $^{14}CO_2$ and enter the biosphere through carbon fixation by photosynthesis. Consumption of plants and animals that eat plants, by humans is mirrored in the ^{14}C level in the DNA of newly dividing cells at any given time. By measuring ^{14}C level in the DNA of hippocampus cells using accelerator mass spectrometry (AMS) and plotting on bomb pulse curve gives the birth date of cells. (The above illustration is adapted from Bergmann O et al., 2015 with permission).

IMPACT OF SUBSTANCE ABUSE ON HIPPOCAMPAL NEUROGENESIS

In this section the effect of alcohol and cocaine on hippocampal neurogenesis is discussed from both animal and human studies.

Alcohol and hippocampal neurogenesis

In the previous sections, the different stage in adult neurogenesis and the overlapping expression of molecular markers was discussed. The newly born cells in the SGZ migrate a short distance to the GCL, where they mature and differentiate into granule cells, form synaptic contact with other granule cells and with axons from perforant pathway, and also establish their axons into the CA3 region in the hippocampus[114,142,181]. There are four main stages in the adult neurogenesis process; proliferation, migration, differentiation and survival, and alcohol is known to affect all these stages to variable degree depending on dosage, intake pattern and duration of exposure[182-184].

Acute binge alcohol exposures in rats diminish neurogenesis[35], but a burst in proliferation and an increase in DCX expression in the DG have been reported to occur after two weeks of abstinence from alcohol[185]. Alcohol administered in moderate doses decreases survival of new neurons but not the proliferation in rats[1]. In non-human primates, non-dependent alcohol self-administration decreased the proliferation, differentiation and survival of progenitor cells in the DG[184]. The time and duration of alcohol intake have differential effect on the process of adult neurogenesis. Further, in animal studies, ablation of neurogenesis using irradiation resulted in enhanced drug taking behavior[186] and voluntary wheel running before and after alcohol intake resulted in reduced alcohol self-administration[187]. However, the latter study did not measure neurogenesis in the hippocampus, although it may be assumed that the physical activity counteracted the decrease in neurogenesis and that this reduced the drinking behavior. Hence, interventions that impacts on hippocampal neurogenesis such as irradiation and voluntary wheel running seemingly can result in changes in drug or alcohol intake[188].

Several studies proposed that new DG neurons might block memories associated with drug seeking and enhance extinction learning i.e. learning induced inhibition of previously acquired memory or responses related to drug use[189,190]. Newly formed neurons in DG exhibit lower threshold of synaptic excitability to induce LTP that plays a major role in memory formation[191]. Thus, the reduction in neuronal turnover or a reduction in adult neurogenesis due to voluntary drug or alcohol administration may result in the conservation of drug related memories[186,192]. However, further studies are needed to more in detail understand the relation between adult neurogenesis and addiction.

Cocaine and hippocampal neurogenesis

Cocaine abuse is often found to be associated with damage in structure and function of brain areas involved in cognition and motor skills[193]. In long-term cocaine abusers there was a reduction in grey-matter volume in hippocampus[194] and prefrontal cortex[195]. Cocaine as a psychomotor stimulant drug, have been found to negatively influence neurogenesis in the DG[2,196]. Longer period of cocaine administration has been shown to decrease cell proliferation[2] without altering the survival and dendritic maturation of newborn DG cells in adult rat hippocampus[196]. Abstinence from cocaine administration in rats normalized the changes in proliferation and enhanced the number of DCX-positive neurons[197].

Moreover, a few postmortem studies on human cocaine addicts have identified dysregulation in hippocampal gene expression, including gene products involved in glutamatergic and GABAergic transmission[198,199], and cell remodeling[200]. These neuroadaptational changes observed in hippocampus were expected to yield anatomical and volumetric changes in the hippocampus. Nevertheless, MRI studies on cocaine addicts in hippocampus has not shown significant volumetric changes in hippocampus[201,202], but some studies using different methodologies have observed some volumetric changes in hippocampus of cocaine addicts[203,204]. However, volumetric changes might not correlate with changes in granule cell numbers in hippocampus of cocaine addicts.

AIMS

The overall aim of this thesis is to gain knowledge about the short-term and long-term effects of alcohol on the neurogenic population and DG granule cell number in the adult human hippocampus. Another aim is to study the cell turnover rates in hippocampus of chronic alcohol and cocaine abusers. The author of the thesis has been assigned, and performed immunohistochemistry to study the effect of alcohol on adult neurogenesis in hippocampus using markers for proliferation, stem/progenitors and immature neurons. The author has also been assigned and performed a stereology based approach in order to further study the chronic lifetime effects of alcohol on granule cells which is the principal cell layer in the dentate gyrus. Finally, the author has also been assigned and performed several of the procedures to birth date hippocampal cells, and hence estimate their turnover rates, using a retrospective ^{14}C dating strategy in chronic alcohol and cocaine addicts, and in control subjects.

SPECIFIC AIMS

- To assess the effect of alcohol abuse on the expression of Ki67, Sox2 and DCX as markers of proliferation, stem/progenitor cells and immature neurons, respectively, both in DG and SGZ. (Paper I)

- To assess the correlation of all the markers with age of the subjects in both alcoholics and control subjects. (Paper I)

- To investigate the density and total number of granule cells, and volume of GCL in subjects with on-going alcohol abuse as compared to control subjects, using a stereological approach. (Paper II)

- To investigate and compare the impact of age on the total number of granule cells, density and volume of GCL in alcohol abusers and control subjects. (Paper II)

- To analyze the turnover rates of hippocampal cells in chronic alcohol and cocaine abusers, and control subjects using retrospective 14C birth dating procedure. (Paper III)

EXPERIMENTAL PROCEDURES

CASE SELECTION AND INCLUSION CRITERIA

In the Paper I, the main aim was to study the on-going effect of alcohol abuse on neurogenesis, since it is known that neurogenesis might be restored during prolonged periods of abstinence[205] and the consequence of associated functional changes may partly be explained by restoration of neurogenesis[206]. The medical history of the deceased study subjects was extracted from different sources such as the police, relatives, medical records, and postmortem findings. The main inclusion criteria for alcoholic subjects in Paper I was daily consumption of large amounts of alcohol or repeated binge drinking episodes for at least 4 weeks prior to death. The choice of this four-week time window was based on the experimental animal studies on primates in which at 6 weeks nearly 84% of all the BrdU labelled cells were DCX positive[153]. However, relatives might not be able to tell the drinking pattern too long time back, and hence we decided to limit the period to 4 weeks.

In the Paper II and Paper III, all the alcoholics were chosen on the basis of long-term alcohol abuse during their lifetime, and the same is valid for cocaine addicts in paper III. To retrieve this information, additional important data sources such as the Swedish national inpatient register[207] and the Swedish prescribed drug register[208,209] were perused to confirm abuse/addiction diagnosis and to assess duration of abuse in addition to the sources mentioned for Paper I. All the alcoholics included in Paper II and Paper III had been drinking heavily for many years and most of the subjects had moderate to severe fatty liver or liver cirrhosis.

None of the subjects included in the three studies were diagnosed with neurodegenerative diseases such as Alzheimer's, Parkinson's or Huntington's disease. The control subjects were free of depression while a few alcoholics were known to have had some depressive episodes during the lifetime but only for short time periods, and not before the onset of the alcohol abuse. Since some alcoholics develop depression, seemingly secondary to their addiction, we think the inclusion of these subjects is justified in order to make this group as representative as possible for chronic alcoholics in the population. Even though there were some alcoholics and control subjects who had benzodiazepines and opiates in the postmortem blood, they had been prescribed these drugs, and none of them were diagnosed with opioid or benzodiazepine dependence.

IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry with specific antibodies against cell and nucleus specific markers was used in Paper I and Paper II to detect cells in DG on the coronal sections of hippocampus. In the Paper I, fluorescent markers were used to study the co-localization of different makers. In the Paper II, microscopy of DAB-stained slides was used since the purpose was to count granule cells with a bright field stereology system.

For the preparation of sections stained with fluorophore-labeled secondary antibodies, the sections were first rehydrated with phosphate-buffered saline (PBS). The sections were then treated with formalin to crosslinking proteins in order to add rigidity to the thin sections for further examination. Blocking buffer containing Triton X-100 and Bovine serum albumin (BSA) in PBS was used after fixation. Triton X-100 is a detergent used to permeabilize the cell membrane for the antibodies to enter the cell and to identify the intracellular localization of the proteins of interest. BSA was used as a blocking agent to reduce background signals due to unspecific binding. The tissue was also treated with blocking buffer with the serum from the same species as the secondary antibodies. The primary antibodies against each protein were added and incubated overnight at 4°C followed by incubation with Hoechst, which is a DNA intercalating agent to mark the nucleus of the cells. Primary against Ki67 (proliferation), Sox2 (stem/progenitor cells), DCX (immature neurons) and NeuN (mature neurons) were used in this study. The secondary antibodies used were conjugated with fluorophores with different colors such as Alexa 488 (green), Alexa 555 and Cy3 (red), which allows for studying co-localization using an epifluorescence microscope. The sections were then mounted with a cover slip using glycerol containing mounting medium. Three to five hippocampal sections from each case were stained with Ki67, Sox2 and DCX, and counted under the epifluorescence microscope and the average density was calculated and analyzed.

For the bright field immunohistochemistry, the tissue sections were directly fixed in formalin. This step was followed by primary antibody treatment as previously described. The endogenous peroxidase activity was blocked by treating the section with hydrogen peroxide (H_2O_2) in the dark. This blocking step is important since the cells in human tissue contain endogenous peroxidases. The sections were then treated with blocking with serum from the same species as the secondary antibody were made in. The secondary antibodies used were conjugated to the enzyme horse radish peroxidase (HRP) which after treatment with substrate 3,3'-diaminobenzidine (DAB) produce a brown precipitate. Mayer's hematoxylin was used as a counter stain to visualize cell nuclei. After the staining procedures the sections were dehydrated in a series of 70%, 95% and absolute alcohol followed by immersion in xylene, and finally embedding in paraffin. Cover slips were mounted using automated machine. The antibody used in this brightfield protocol was NeuN, a nuclear protein marker that homogenously stains the nuclei of mature neurons. The sections were quantified regarding

NeuN positive cells by a morphometric method with optical fractionator of stereology explained in the following section.

MORPHOMETRIC QUANTIFICATION

The total number of granule cells in the sample of GCL studied in paper II was estimated using a morphometric method adopting stereological principles based on the optical fractionator method[172]. From the mid-portion of hippocampus at the level of the lateral geniculate body a coronal block of the tissue, approximately 4-5 mm thick, was collected and 100 sections, with a thickness of 20 μm thick were prepared using a cryostat. The sections used for stereological analysis was selected from a 2 mm portion from the anterior part of the coronal block. With a randomly picked first section, an additional four sections at constant interval from the first were selected for the study and stained for NeuN as previously described. The GCL was delineated using the stereological instrumental set up and optical fractionator estimation of NeuN positive granule cells was accomplished by meander sampling function of a computer assisted stereological tool (CAST) software program. A sampling scheme was designed so that from each case 150 to 200 neurons were counted per subject according to the guiding rules of the optical fractionator method[210]. The area of the counting frame and the percentage of sampling factor was optimized to achieve the required number of counting from each case. Since the thickness of sections were less than 20 μm all the counting was performed along the thickness with no use of guard heights at the top and bottom. The total number of granule cells, the density and volume of GCL were calculated using the following equations,

Total number of neurons (N) in the mid-portion of the hippocampus was calculated by

$$N = \Sigma Q^{-} \times 1/TSF \times 1/ASF \times 1/SSF$$

The volume of the GCL (V) was calculated using the relation,

$$V = T \Sigma \text{ Area (GCL)}$$

Number per volume (N_v) or density of granule cells were calculated by

$$N_v = \Sigma Q^{-} / V(\text{disector})$$

The factors used in all the above mathematical equations are explained as follows: ΣQ^{-} is the number of granule cells in a known volume fraction of the GCL, ASF (Area Sampling Fraction) is the proportion of sampled area of GCL expressed in percentage, Area (GCL) is the measured area of the GCL of each section, T is the mean section thickness of each case measured using microcator, TSF (Thickness Sampling Fraction) is the disector height (h) to thickness ratio, and SSF is section sampling fraction - in this study it is 1/20 - selected in the interval of 20 sections from 100 sections made on each block of hippocampus.

CELL TURNOVER STUDIES – ^{14}C BIRTH DATING

Nuclei Isolation and FACS sorting

The whole hippocampus of both sides was collected. The isolated hippocampal tissue was thawed and homogenized using a Dounce homogenizer with lysis buffer containing sucrose and Triton X-100, where sucrose stabilizes the nuclei and Triton X-100 as a detergent helps to lyse the hippocampal cells. The homogenized mixture was carefully added on the top of a sucrose solution of higher density to make a molecular sieve followed by centrifugation to isolate the hippocampal nuclei from the suspension. Isolated pellets containing nuclei were resuspended in nuclei storage buffer for consecutive flow cytometric analysis.

Isolated nuclei were stained for the anti-neuronal nuclear antigen NeuN antibodies conjugated with Alexa 647 in order to separate neuronal and non-neuronal nuclei using Fluorescence activated cell sorting (FACS). The forward and side scatter function of FACS sorting was initially used to separate the whole nuclei on the basis of size and granularity, respectively. The whole nuclei were sorted regarding NeuN positivity and the fractions collected in separate tubes followed by purity measurement. The nuclei suspension was centrifuged and the pellet was collected in a special tube free of carbon contamination for DNA extraction. This nuclei isolation and sorting was then followed by DNA purification and carbon-14 quantification by AMS was performed in Paper III for studying cell turnover rates of the hippocampal cells.

DNA Purification

DNA was isolated from the nuclei by lysing the isolated nuclei with nuclei lysis buffer containing sodium salt of EDTA followed by treatment with DNA lysis buffer (containing SDS) and Proteinase K treatment[211]. Proteinase K was used to lyse and separate the protein

contamination from DNA. The DNA suspension now free from protein was treated with RNase cocktail to lyse and remove the contamination of RNA. The DNA in the suspension was precipitated using absolute ethanol and washed in a mixture of ethanol and sodium chloride. The finally precipitated DNA was resuspended in DNase- and RNase- free water and measured for purity using UV spectroscopy (NanoDrop).

Accelerator Mass Spectrometry

The purified DNA suspended in water was lyophilized to dryness and converted into graphite in order to quantify the carbon-14 levels in DNA. For this conversion, excess copper oxide was added to the dried DNA and the tubes were evacuated and sealed using a high-temperature torch. The sealed tubes were treated at high temperature at 900°C to convert all the carbon into CO_2. In a separate reactor the formed CO_2 from each sample after heat treatment was reduced to graphite Zink using iron as a catalyst. Graphite targets were measured at the Tandem Laboratory at Uppsala University[212]. Stable isotope ratio mass spectrometry was used to measure the carbon-13 (^{13}C) levels, another carbon isotope, and a correction factor using the ^{13}C values was applied to each sample. The CO_2 samples obtained after the high temperature treatment was split into large and small CO_2 samples with a concentration of >100 µg and 10 µg, respectively. The corrections for background contamination and measurement error was determined and applied for all samples measured[213,214]. The ^{14}C data for each individual sample was reported as decay-corrected $\Delta^{14}C$ and fraction modern.

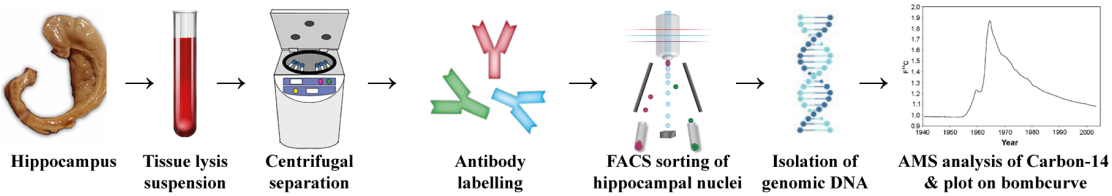

Figure 4: A schematic representation of the ^{14}C birth dating procedure. Hippocampus tissue was weighed and lysed into a cell suspension. The nuclei were separated using centrifugation and labelled by fluorophore conjugated NeuN antibody. The neuronal and non-neuronal nuclei were sorted using FACS followed by genomic DNA extraction from the nucleus. ^{14}C level from the nuclei was measured using AMS and the cells were birth dated on the bomb pulse curve as shown.

STATISTICAL ANALYSIS

In **Paper I**, Mann-Whitney U-test was used to analyze the significance between mean index values of markers, and the data are expressed as mean ± SEM. Spearman's rank correlation was used to analyze relationship between each marker's index values and continuous variables. A p-value < 0.05 was considered statistically significant.

In **Paper II**, the distribution of data in each group was found not to be different from normal distribution using Kolmogorov-Smirnov test. The difference between groups regarding total number of granule cells, volume of GCL and the density was analyzed using one-tailed unpaired t-test with Welch's correction. A p-value < 0.05 was considered significant. All the variables studied were analyzed for covariance (ANCOVA), by taking brain pH, age, total PMI, brain weight and gender as covariates. A linear regression analysis was applied to assess the effect of age of the subjects on each of the analyzed variables.

In **Paper III**, the general difference in turnover rate of neuronal and non-neuronal cells between groups were estimated using Wilcoxon rank-sum test. The effect of age and alcohol duration on turnover was analyzed using linear regression analysis. Non-linear least square calculations and Markov Chain Monte Carlo (MCMC) algorithms were used to estimate best global parameters for each scenario for the neuronal and non-neuronal populations. A p-value < 0.05 is considered significant in all analysis.

ETHICS

In the Paper I and Paper II, brain samples from deceased donors were collected by KI-Donatum, a core facility that handles selection of donors, retrieval of medical history and collection procedures in collaboration with the forensic medicine department in Stockholm. In Paper III, brain samples were obtained both from KI-Donatum and from the University of Miami Brain Endowment Bank. All the studies included in the thesis were approved by the ethical review boards in Stockholm and Miami, respectively.

Ethical approval numbers:

Paper I: EPN Dnr 2010/313-31/3

Paper II: EPN Dnr 2010/313-31/3 and 2018/689-32

Paper III: EPN Dnr 2010/313-31/3, 2018/689-32 and University of Miami, IRB: 19920580 (CR00006788)

RESULTS AND DISCUSSION

PAPER I

Effect of alcohol on the neurogenic cell pool in SGZ

The effect of on-going alcohol abuse on proliferating cells, stem/progenitor cells, and immature neurons in the alcohol abusers and control subjects was studied using immunological stage specific markers of adult neurogenesis on postmortem human subjects.

The main finding of the study was a significant reduction in Sox2 and DCX positive cells in the SGZ of alcohol abusers as compared to age matched control subjects (Figure 5B and 5C). DCX has been considered as a marker for immature neuron in this study; DCX has been used in many studies as a marker for immature and migrating neurons in the adult hippocampus[141,163]. The finding that alcohol could affect DCX-IR neuronal cell, representing immature/dividing cells is fairly straightforward to show the detrimental effect of alcohol on a neuronal cell in the hippocampus as previously observed only in glial cells of alcohol abusers[92].

In addition to the decrease in DCX-IR in SGZ, the Sox2-IR cells were more prominently reduced in SGZ than DCX. As Sox2 is marker for stem/progenitor cells in the SGZ, it implies that alcohol may affect early rather than late developmental stages in adult neurogenesis. The decrease in DCX could therefore be considered as secondary effect of alcohol induced reduction of the stem/progenitor cells in the SGZ.

Most of the DCX positive immature neurons were also positive for NeuN, a marker for mature postmitotic neurons as used in previous study[215]. This implies that alcohol could have secondary effect on survival and integration of newly matured granule cells. However, the co-localization of DCX and NeuN was not systematically quantified in this study.

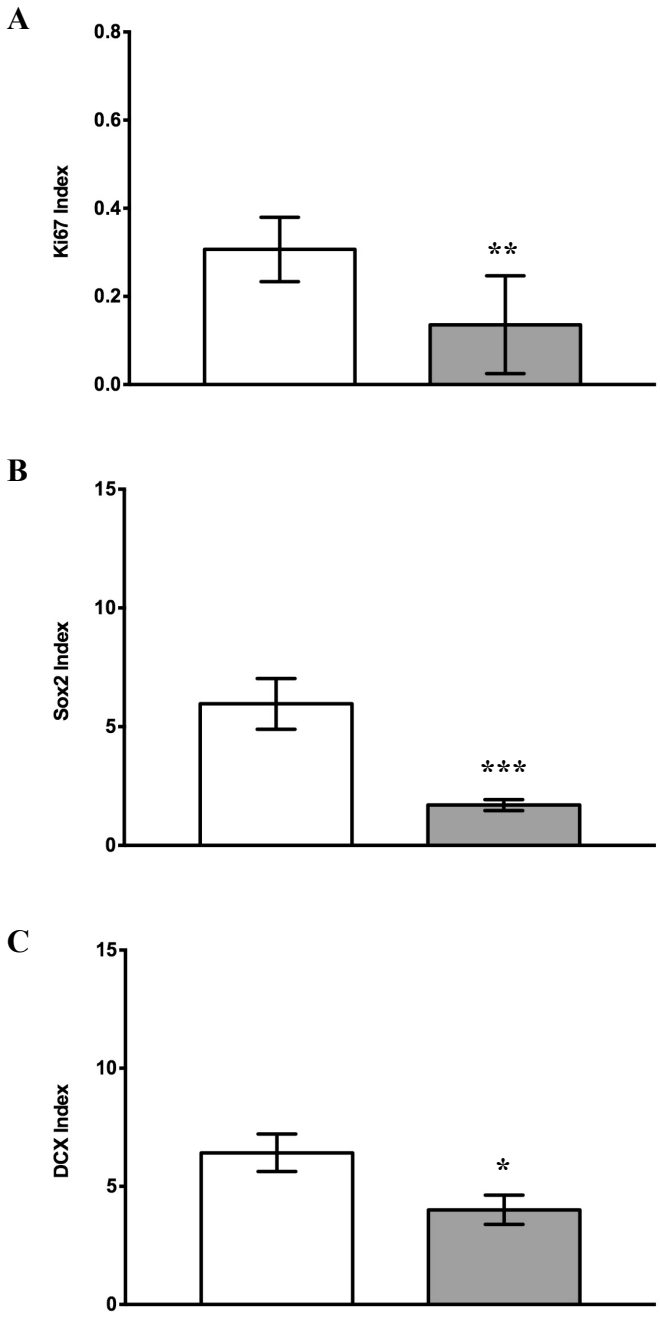

Figure 5: Immunopositivity of markers in SGZ expressed as number of positive cells per unit area of GCL (mm^2) for each subject. Ki67 (A), Sox2 (B), and DCX (C) were significantly reduced in alcohol abusers compared to control subjects. Bars represent mean index for control (white) and alcoholics (grey) with error bars (±SEM). *p<0.05, **p<0.01, ***p<0.001 (Mann Whitney U-test).

The immunoreactivity of Sox2 in the SGZ is interpreted as stem/progenitor cells in this study. However, Sox2 is also expressed by some astrocytes with unknown functional involvement in adult neurogenesis[216]. As GFAP is widely used as a marker for mature astrocytes in the adult brain, the co-labeling of Sox2 with GFAP was studied, and almost all Sox2-IR cells were found to be negative for GFAP in all the areas of the DG studied. This implies that the Sox2 cells studied most likely rather represent stem/progenitor cells than astrocytes and hence that the reduction of Sox2-IR reactivity actually represent a reduction of stem/progenitor cells in the SGZ, considered as the neurogenic niche in the hippocampus.

The density of Ki67-IR cells in the SGZ was lower in alcoholics as compared to controls (Figure 5A). This finding is in line with reports of a decrease in density of Ki67-IR cells in rodent model of binge drinking[217,218] and also with reported heavy binge alcohol effect on non-human primates[184]. In addition, alcohol binge drinking is found to reduce the cells expressing Ki67 and Sox2 in non-human primates[184]. In this study, a small portion of Sox2-IR cells in the SGZ show positive labeling for Ki67. However, the proportion of co-immunoreactivity between Ki67 and Sox2 was not systematically studied in this study.

Effect of alcohol on the neurogenic cell pool in DG

The fate/quantification of immunoreactivity of all the markers in DG was also studied, as all of the markers adopted to use in this study has been reported to be expressed in several brain regions[219-221]. In addition to SGZ the DG includes GCL and ML. The delineation used for ML was set to 100 μm as used to define SGZ in this study. The immunoreactivity of Ki67, Sox2 and DCX is found decreased in alcoholics compared to controls in the whole DG (Figure 6).

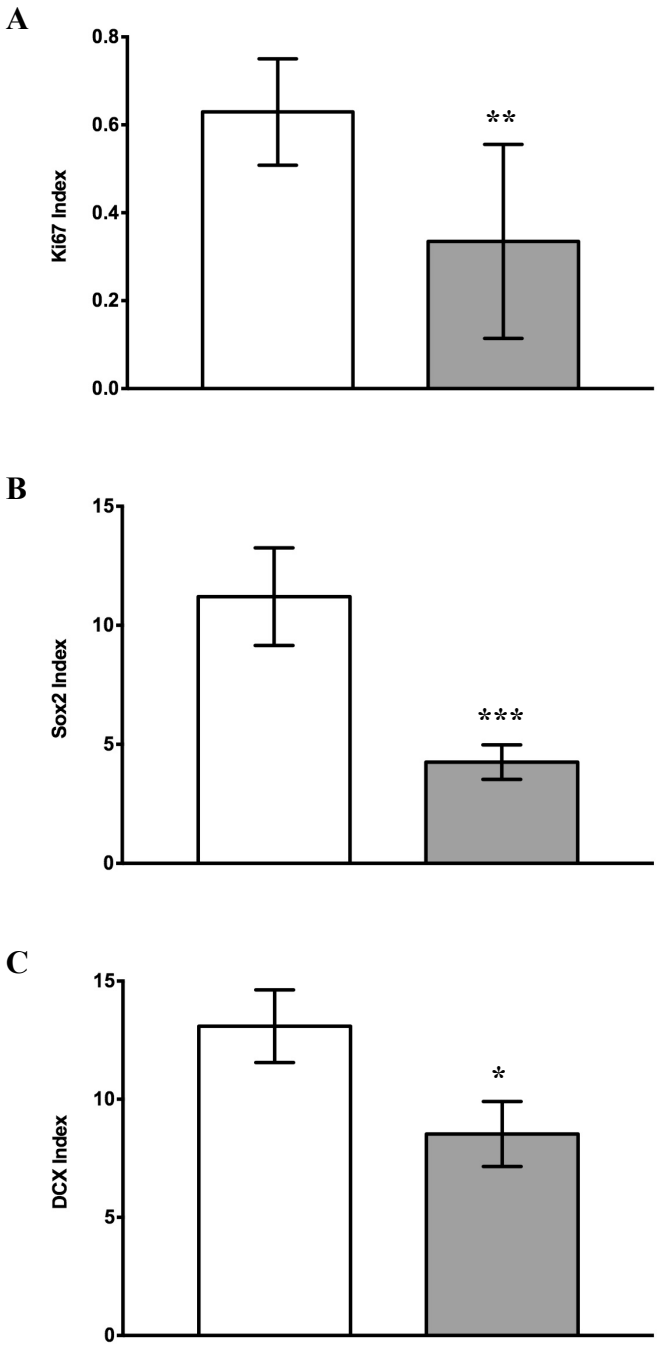

Figure 6: Immunopositivity of markers in DG expressed as number of positive cells per unit area of GCL (mm^2) for each subject. Ki67 (A), Sox2 (B), and DCX (C) were significantly reduced in alcohol abusers compared to control subjects. Bars represent mean index for control (white) and alcoholics (grey) with error bars (±SEM). *p<0.05, **p<0.01, ***p<0.001 (Mann Whitney U-test).

Although all the markers studied were observed in ML in both groups, Sox2 and DCX were significantly decreased in ML of alcoholics compared to controls. However, role of Sox2 and DCX in ML has not been explored systematically in studies on adult neurogenesis. In animal studies, different cell types have been observed in ML, such as interneurons specific for ML, molecular layer perforant pathway (MOPP) cells and far migrated immature neurons[48,153,219,222,223]. MOPP cells were found to innervate newly generated granule neurons, which is linked to dendritic sprouting associated DCX expression[221]. Sox2-IR cells in the DG were negative for GFAP as observed in SGZ in both alcoholics and control subjects, and they may represent progenitor cells (Type 2a or Type 2b) as proposed by Knoth et al., 2010[141].

Further the DCX-IR in the whole DG was significantly decreased in alcoholics as observed in SGZ alone, also in line with rodent studies on binge and chronic ethanol exposure[218,224]. Co-staining of DCX with PSA-NCAM revealed a larger co-expression of the two markers in the DG compared to previous human studies[215]. The topological distribution of immunoreactivity of the markers in the SGZ and DG showed a general reduction in alcoholics compared to control subjects, and hence not at a certain distance from the GCL. Hence, these results suggest that alcohol reduces cells expressing Ki67, Sox2 and DCX in all the layers of DG and thus not only in SGZ of the hippocampus.

Correlation of markers with age of the subjects

In this study we found a negative correlation of Ki67-IR density with age of the subjects in control group. However, there was no correlation of Ki67 with age in alcoholic subjects, and neither did Sox2-IR or DCX-IR show correlation with age (Figure 7). Other human studies have reported a modest decline in neurogenesis during aging[119,141], especially decrease in DCX positive cells with age in humans[141]. The reason why we did not observe a correlation with age could be due to limited age range, limited number of cases and a considerable level of variability between cases. In addition, studies in rodents have shown an exponential decrease of neurogenesis with increasing age, and the steepest change takes place during 3-6 months of age to reach a low and constant level thereafter[225]. Extrapolated to the human lifespan, it might be expected that the detectability of differences in neurogenesis (using different markers) should be highest in the youngest subjects[141]. The majority of the subjects in our study are adult within a somewhat older age range (median age in controls was 56 and for alcoholics 55 years); this could also explain the low variation in index values of the markers used.

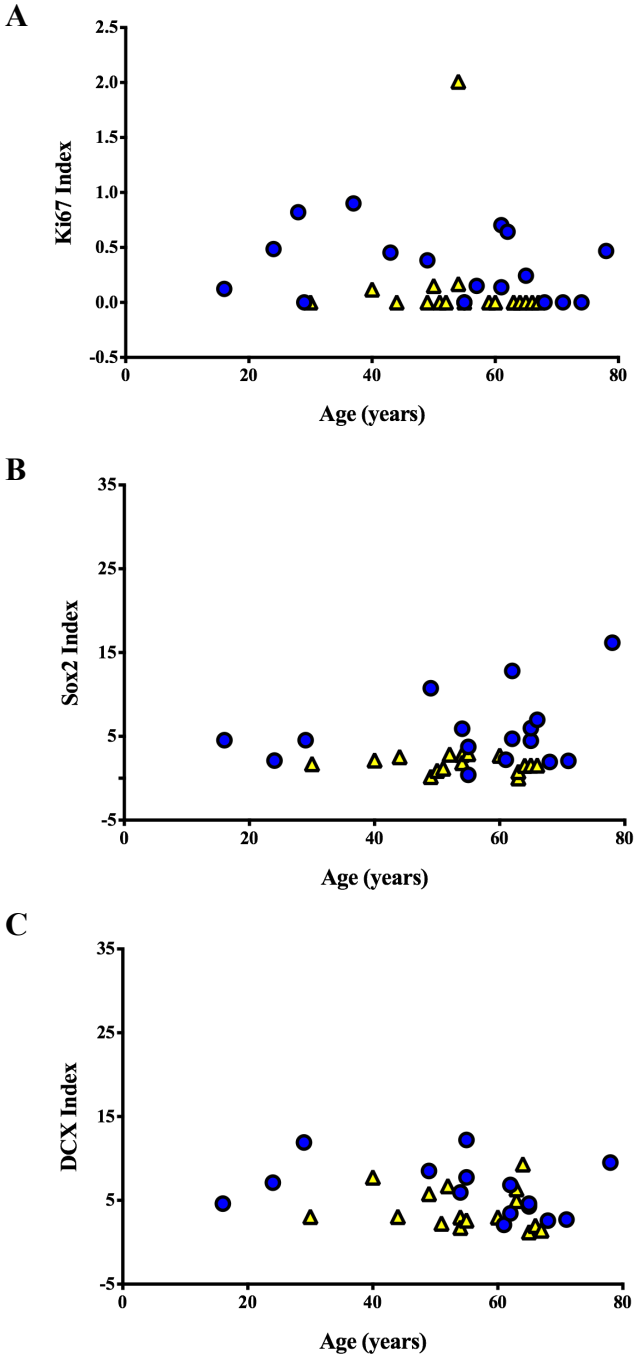

Figure 7: Correlation plot shows markers relationship with age of the subjects in SGZ. (A) Ki67 shows significant decrease with age in controls (p=0.0470) and in all subjects (p=0.0242), but we have not observed significance in alcohol abusers (p=0.2917). There was no significant correlation between either Sox2 or DCX with age of the subjects in both alcohol abusers (yellow, triangles) and control subjects (blue, circles).

PAPER II

Since there was a reduction in putative stem/progenitor cells and immature neurons (Paper I), we wanted to find out if this reduction in subjects with an on-going alcohol abuse would over time result in a reduction of the granule cells in the GCL of hippocampus. If so, this could be a stronger support for the notion that impairment of neurogenesis by alcohol can have an effect on memory and learning, which are functions dependent on the GCL granule cells, including those newly formed, rather than on the cells involved in neurogenesis. Previous studies however failed to observe granule cell loss in hippocampus[92,226], which could be attributed to a limited number of cases, limited age range of subjects, less defined inclusion criteria of subjects used and the use of non-specific neuronal staining to quantify mature granule cells.

Effect of alcohol on granule cell number and GCL volume

The difference in number of granule cells between control and alcoholic subjects were estimated using optical fractionator of stereology. The number of granule cells and the volume of GCL were significantly reduced in alcoholic subjects compared to control subjects (Figure 8A and 8B). The volume of the GCL was reduced to smaller degree (20%) than the number of granule cells (52%) and hence the density of granule cells was also significantly reduced in alcoholics as compared to controls (Figure 8C). The density of granule cells estimated in this study was found to be comparable to conventional stereological studies[174,175]. Since in this study we have used only a mid-portion of hippocampus, not the whole hippocampus as in standard stereological studies, this density correlation with other studies adds strength to the current study.

There are several reasons for using the selected portion of hippocampus; this region is the anterior part of the posterior hippocampus, which is known to be involved in the retrieval of episodic and spatial memories[68,227]. This part of the hippocampus is anatomically identifiable, located at the level of lateral geniculate body, and represents the posterior part of the hippocampus, known to be involved in episodic memory retrieval and suitable to study the effects of addiction [228]. This part of the hippocampus was also studied in several other studies with different purposes[79,229].

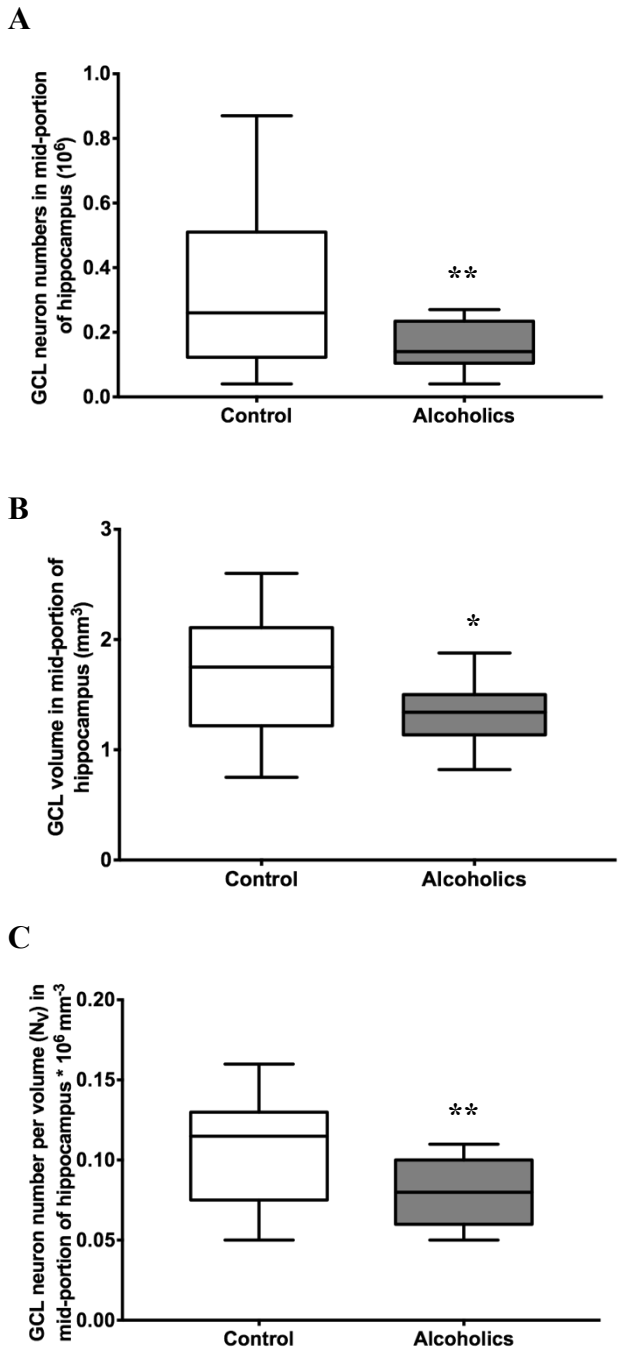

Figure 8: Box plot shows the difference in total number of granule cells (A), GCL volume (B) and density (C) between control (white) and alcoholics (grey). There is a significant difference between all the three variables quantified using a morphometric procedure based on stereological principles. *p<0.05, **p<0.01 (one-tailed unpaired t-test with Welch's correction).

The reduced number of granule cells in alcoholics observed in this study may not only be due to a decrease in stem/progenitor cells and immature neurons as observed in paper I. It could also be due to apoptotic and necrotic granule cell death as observed in binge exposure of alcohol for prolonged time in animals[230-233]. However, in humans, a downregulation of activated caspase-3, a marker of apoptosis, has been shown, suggesting that chronic alcohol abuse may not activate apoptotic machinery in humans[234]. We have also performed an immunostaining using apoptotic marker cleaved caspase-3 co-labeled with NeuN, but failed to observe positive staining for cleaved caspase-3 in GCL. Nevertheless, cleaved caspase-3 staining was only used on a few cases in this study.

There are only two possible explanations for this observed granule cell loss in alcoholics. The episode of overconsumption of alcohol may result in cell death or the impaired neurogenesis may impair the incorporation of newly matured granule cells in alcoholics. And of course, a combination of these two possibilities may lead to net loss of granule cells.

The granule cell loss in alcoholics was observed in the mid-portion of the hippocampus in which the impairment of stem/progenitor cells and immature neurons was also observed in Paper I. Several animal experiments reporting reduction of immunoreactivity of stem/progenitor cells and immature neurons by alcohol also studied this part of hippocampus[35,218,224,235]. A correlation between dentate gyrus regenerative capacity and the number of granule cells was proposed in a seminal study on patients with intractable epilepsy[229], that could add strength to the notion that neurogenesis directly regulates granule cell numbers in humans. These observations further lend support to the notion that alcohol reduces the addition of new granule cells and thereby affecting the homeostasis between new cell addition and natural cell loss that may result in net loss of granule cells observed in this study.

Age effect and other confounding factors

We have also found that age has significant impact on the total number of granule cells and volume of GCL in the control subjects used in this study (Figure 9). This is line with previous human studies in which age related modest decline in both neurogenesis and neuronal cell turnover in the hippocampus has been observed[119,141]. However, the impact of age was not significant in alcohol abusers which may due to the fact that most of the subjects in this cohort was between 40 to 70 years of age. Age has significant impact on granule cell numbers and volume of GCL when both alcohol abusers and controls were pooled, which implies that age might have impact on alcohol abusers as well. Additionally, in this study one of the subjects aged 23 in the alcohol cohort showed low granule cell number and volume of GCL which suggest that alcohol could have larger effect on neurogenesis and cell turnover in early adulthood than at later stage in life.

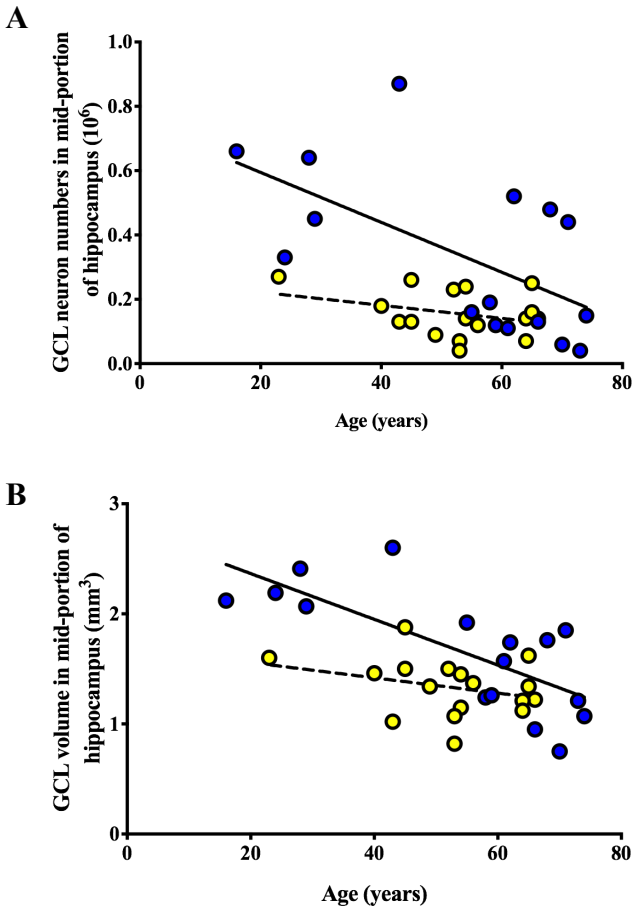

Figure 9: Granule neuron number and volume of GCL in the hippocampus region studied with age of the subjects. (A) Age related decline in granule cell number in control ($r^2 = 0.35$; n = 16; $p < .02$) and alcoholics ($r^2 = 0.10$; n = 17; $p > .05$). (B) Age related decline in volume of GCL in control ($r^2 = 0.53$; n = 16; $p < .002$) and alcoholics ($r^2 = 0.09$; n = 17; $p > .05$). Control – Solid line and blue circles, Alcoholics – Dashed line and yellow circles.

The current study also has certain confounding factors that need to be considered. The alcoholic group in this study includes four subjects with depressive episodes, but which we think this proportion may be representative of alcohol abuse population[236]. As depression is known to affect neurogenesis in animal species studied[237,238] and also in humans[49], we have checked for the significance of our results by excluding those cases, and a significant reduction of granule cells was still observed in the alcoholic group. Further, most of the subjects with antidepressants were prescribed for selective serotonin reuptake inhibitors (SSRIs), and some of them also had lower concentration of these drugs in the postmortem blood. In general, the effect of antidepressants on proliferation is mainly observed in the head of hippocampus, and SSRIs have much less effect on proliferation than tricyclic antidepressants[49].

In conclusion, there are several factors that may influence the number and density of granule cells in GCL of the adult human hippocampus, but as explained in this section, there is limited support for the assumption that these factors would have influenced the results to any larger extent.

PAPER III

The average turnover rate of neurons and non-neurons in the human hippocampus using retrospective birth dating based on incorporation of nuclear test bomb derived ^{14}C into the nucleus of dividing cells has previously been reported for subjects of different age[119]. From experimental studies it is appreciated that several factors influence neurogenesis in hippocampus. In this study we focused on the possible influence of alcohol and cocaine abuse on the turnover rate in hippocampus by collecting hippocampus from well-characterized deceased donors with either long-term alcohol or long-term cocaine abuse, as well as from controls with no history of abuse. Subjects with major psychiatric disorders were excluded.

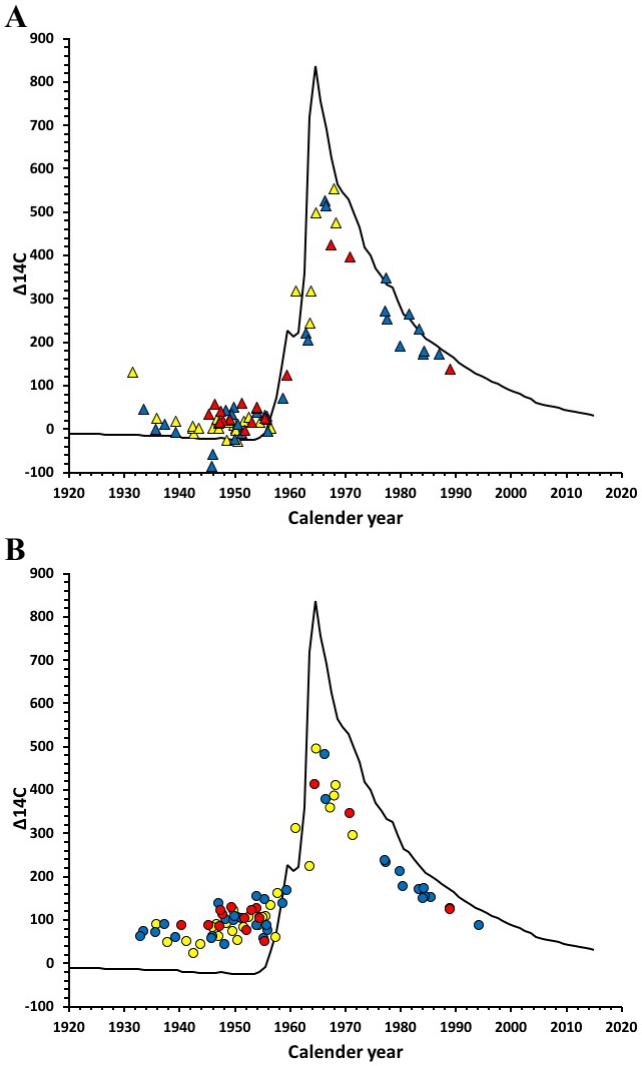

Figure 10: The ^{14}C concentration of genomic DNA from (A) neuronal (triangles) and (B) non-neuronal (circles) cells in Control (blue), Alcohol abusers (yellow) and Cocaine (red) demonstrate postnatal cell turnover in subjects born before and after the bomb spike.

Turnover of neurons and non-neurons in control and alcohol abusers

In this study, mathematical modeling on neuronal and non-neuronal cell population was performed with the following three assumptions: a constant turnover throughout the lifetime of the individual; a specific effect of duration of alcohol abuse on cell turnover, and by a complex model in which a separate turnover rate for pre- and post- onset period of alcohol abuse was calculated.

In general, we observed a turnover of both neuronal and non-neuronal cells after birth of the individual in all the three groups studied in (Figure 10). This is in line with the previous findings that there is a turnover of neurons and non-neurons in the adult human hippocampus[119]. Chronic cocaine addicts showed turnover rates of neurons and non-neurons that were indistinguishable from controls. Alcoholics did not either differ from controls regarding the turnover rate for the non-neuronal population, but showed a lower turnover rate of neurons. However, the alcoholics were older than the controls and when the turnover rate was corrected for age, this significance disappeared. Hence even if the decline in turnover rates of hippocampal neurons in the human brain is seemingly not as steep as in animals, this change is still strong. Alcohol addiction may start late in life, and indeed in many of the cases included the onset was at the age of 40 or later. Hence, the possible reduction in neurogenesis by alcohol may be difficult to detect in subjects who developed addiction late in life, when the turnover rates are lower.

From the box plots in Figure 11A it may look as the alcoholics have a lower turnover rate of neurons than controls and cocaine addicts, however this difference is not significant. There are certain factors that may explain the results and basically, we propose two possible hypotheses:

1) No difference in turnover rate between alcoholics and controls
2) Reduced neuronal turnover in alcoholics

In Paper II, we have observed more than 50% reduction in total granule cell number in DG of alcoholics compared to controls[239] using a morphometric method based on stereological principles. In another histological study, Bengochea et al. identified a 50% reduction in CA pyramidal cells in alcoholics along with a 35% reduction of granule cells[91]. The neuronal loss observed in the hippocampus in these studies may be due to the loss of both newly formed neurons (in DG) and old neurons (both in DG and CA). In the human hippocampus about 50% of the neurons are granule cells and the rest CA neurons; CA1 region predominating. We have collected a small section for histological examination from most of the hippocampi used for retrospective ^{14}C birth dating and also found that around 50% of the neurons are granule cells. Since the pyramidal CA neurons are not known to renew, we can assume that all these cells are approximately of the same age as the individual. Regarding the granule cells, the reduction in alcoholics might be partly due to a reduced neurogenesis and partly due

to a removal of old cells. Hence it can be assumed that the majority of cells lost are old. This means that the neurons actually dated at the ^{14}C AMS analysis are expected to include a higher proportion of newer cells that have not been removed, and therefore it could rather be expected that alcoholics should show a higher turnover rate than controls. Hence, the finding that alcoholics rather showed lower than higher neuronal turnover rate than controls could be due to a reduction of neurogenesis by alcohol. However, the mathematical models used were not designed to correct for a variable cell loss.

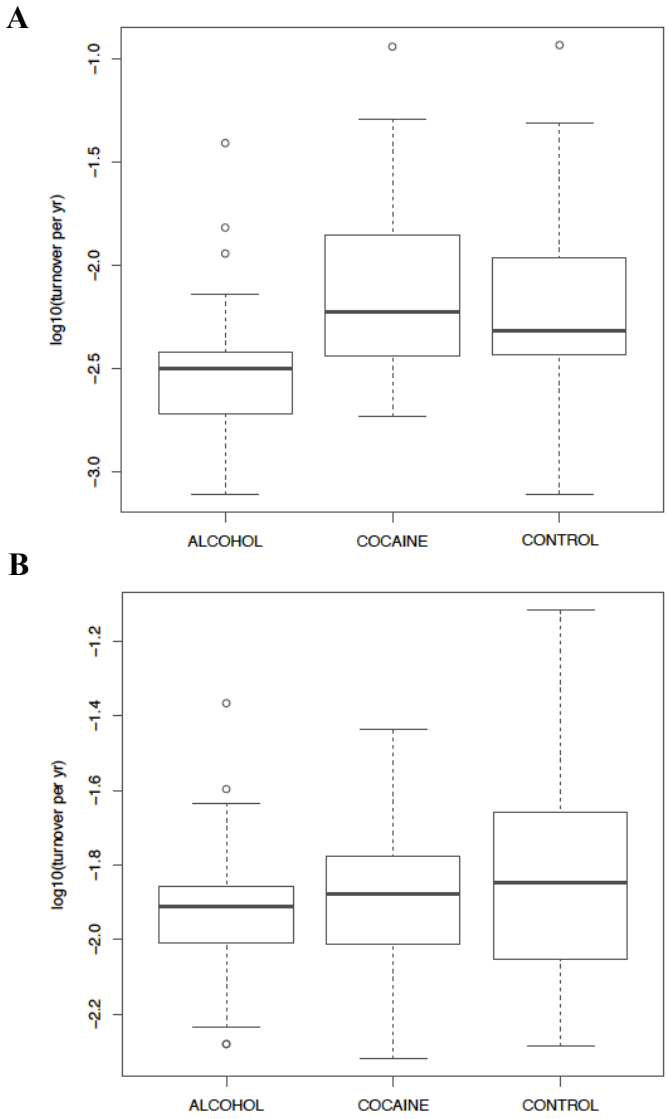

Figure 11: Turnover rates of neuronal and non-neuronal hippocampal cells in the three groups studied. (A) Turnover rates of neuronal cells in alcohol, cocaine and controls subjects. (B) Turnover rates of non-neuronal cells in alcohol, cocaine and control subjects. No significance has been observed between groups.

Effect of age on turnover rates

The age of the subjects is the most important factor that influences the turnover rates. This is also in accordance with experimental data[240,241]. The average age of alcohol abusers and control subject was 53 and 47 years, respectively. A strong effect of age on turnover rates was observed both for neuronal and non-neuronal populations. In this study, the effect of alcohol was not observed when control for age factor was applied using all the three different models used to analyze the turnover rates. Since the majority of alcohol abusers included in this study were older than control subjects the lower turnover rate in alcoholics could be explained by older age. The average age of the subjects in cocaine abusers is 43 years which might explain that they have neuronal and non-neuronal turnover rates are indistinguishable in cocaine when compared from controls.

Effect of cocaine on turnover rates

The cocaine addicts were very similar regarding turnover rates for both NeuN positive and NeuN negative cell populations (Figure 10A and 10B). This was found both when constant turnover rates and when 2POP scenario was applied on the data. The cocaine addicts were in general younger that the other groups, and it is thus possible that some difference may be observed if older cocaine addicts had been included. This is in line with animal studies in which cocaine administration for a longer period resulted in decreased proliferation[2], but not altering the survival and dendritic maturation of new cells in the rat hippocampus[196]. In human studies on cocaine abuse there has been a decrease in grey matter in prefrontal cortex[195], but some imaging studies also shows hippocampal volume deficits in chronic cocaine abuse[194]. Our results do not support that chronic cocaine abuse affects cell turnover in the adult human brain. However, even cocaine addicts may have an increased loss of neurons, which could influence the calculated turnover rates.

In conclusion, in this study no significant difference was observed regarding the effect of alcohol or cocaine abuse on neuronal cell turnover in hippocampus. For alcoholics we cannot exclude that the turnover rates are actually lower than controls if the loss of old neurons are taken into account. For all groups many other factors, related or unrelated to substance abuse may impact neurogenesis, including physical activity, which is very difficult to assess.

CONCLUSIONS AND FUTURE PERSPECTIVES

Many experimental studies have suggested a link between hippocampal neurogenesis and abuse, and both alcohol and cocaine regimens have been reported to impair neurogenesis. In humans, several experimental methodologies cannot be applied. Using three different approaches, we have provided new knowledge in which adult neurogenesis might have a critical role in promoting the development of alcohol abuse or addiction (Figure 12). We show that alcoholic subjects with on on-going heavy drinking has a reduced number of proliferating cells, putative stem/progenitor cells and immature neurons in hippocampus. We have also found that the number of granule cells in the dentate gyrus is profoundly reduced in chronic alcoholics compared to controls. Furthermore, we have also demonstrated the relationship between lifetime or years of abuse of alcohol and the cell turnover in the hippocampus of humans which has not been previously studied.

Conclusions from the individual papers in this thesis are as follows:

Alcohol affect proliferation, stem/progenitor cells and immature neurons in the SGZ and DG in the adult human hippocampus. In alcohol abusers, a profound reduction of Sox2-IR cells (stem/progenitor cells) was found, and also a significant reduction of DCX-IR cells (immature neurons), which may be due to the loss of Sox2-IR cells. Further, the proliferation (Ki67-IR cells) was reduced in alcoholics, but also significantly reduced with age in the control subjects. This adds support to the known decrease in adult neurogenesis with age. In this study all the markers shown a wide interindividual variation which is line with other human studies and which is expected given the many factors that may influence neurogenesis at any given time. (Paper I)

In chronic alcohol abusers, there is a substantial decrease in total granule cell number and volume of GCL. The density difference in granule neurons of alcoholics is mostly attributed to the reduction in total number of granule cells since the decrease in volume of GCL is proportionately less compared to substantial decrease observed in the total number of granule cells in the mid-portion of hippocampus. There is also a significant correlation between the total granule cell number and volume of GCL in alcohol abusers and control subjects. The density of the granule cells in this study is comparable to previous stereological studies on humans. Both total granule cell number and volume of GCL show a negative correlation with age, which is line with previous human studies. (Paper II)

In chronic alcohol abusers, we found a significant lower neuronal turnover rate in hippocampus, but this significance was lost when the results were corrected for age. Hence, age has a strong impact on the turnover rate of the neurons in the human hippocampus. In

cocaine abusers the turnover rate of neurons and non-neuronal cells was indistinguishable from control subjects. (Paper III)

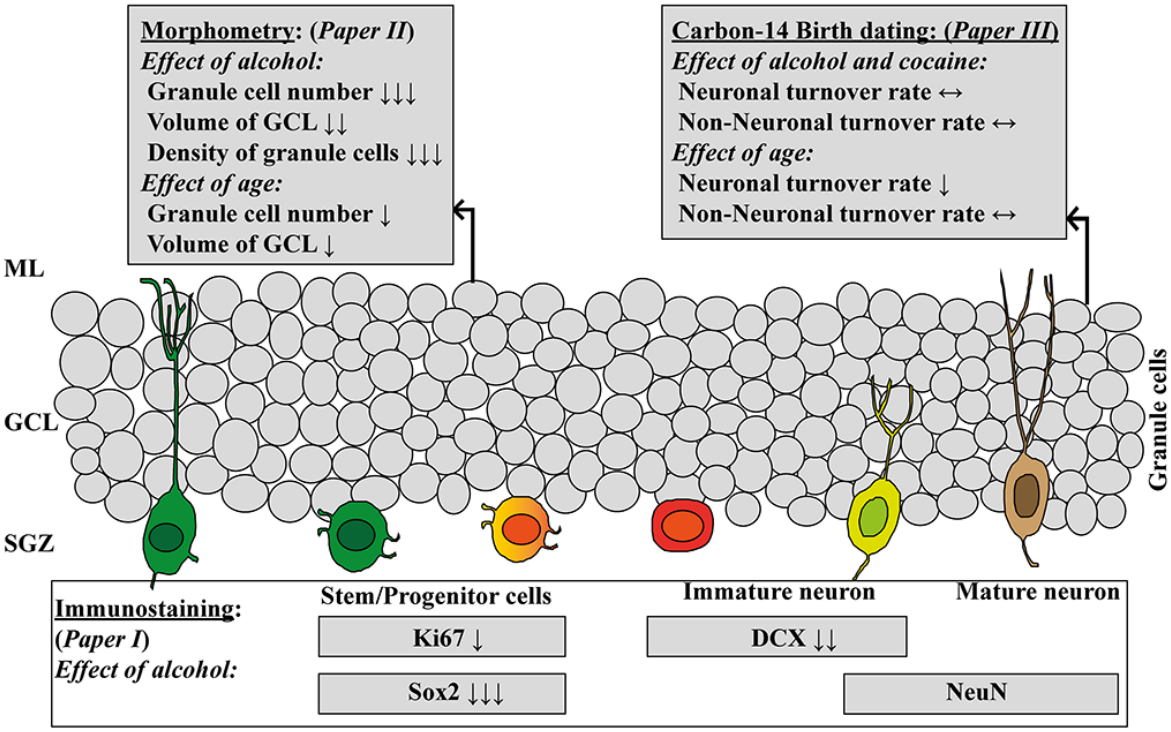

Figure 12: Graphical summary of the thesis showing the impact alcohol and cocaine abuse on hippocampal cells in humans using three different approaches.

Based on the results presented in the thesis it would be interesting to quantify the immunoreactivity for the markers of neurogenesis more specifically by systematically investigate different combination of markers. In the present studies, we focused on markers for only two cell types involved in neurogenesis, but the effect of alcohol on several steps in neurogenesis might be possible to determine if more markers are used. For example, the quantification of colocalization of DCX with PSA-NCAM could narrow the immature cell stage that alcohol could affect more specifically. Double staining of DCX-Ki67 and Sox2-Ki67 would also be important to study in order to determine the specific effect of alcohol on the proliferation of putative neuronal stem cells. For the colocalization studies care should be taken to have short warm time, short fixation time and storage time in the freezer since certain marker proteins may be degraded during the postmortem interval. Further it would be interesting to look at the effect of alcohol on anterior, mid and posterior hippocampus using stereology in order to evaluate the possible regional differences in proliferation and related

deficits the dentate gyrus of alcoholics. Moreover, studies should also include quantification of CA-pyramidal neurons and glial cells in the hippocampus using stereology on chronic alcoholics to further assess the lifetime effect of alcohol. Moreover, it would be interesting to compare the densities of astrocytes and microglia in DG of alcoholics and control subjects. The future studies that relates alcohol abuse with adult neurogenesis in the hippocampus in humans should also focus on expression of different proteins and it might be interesting to particularly study cell cycle proteins with different methodologies. It could be also interesting to compare alcoholics with long period of abstinence with those with an ongoing abuse in order to find out if there is a restoration of the neurogenesis in the human hippocampus as has repeatedly been observed in animals. Even if alcoholics may drink heavily each day for extended periods, they typically also abstain from alcohol for different reasons for shorter or longer times. The neurogenesis in alcoholics during abstinence is unknown. Using ^{14}C birth dating procedure, studying the turnover rates of DG granule cells and CA-cells separately would be most interesting in order to confirm non-renewal of CA neurons, but in particular for better pinpointing the dynamics of the granule cells. We have tried to analyze DG separately, but typically the number of cells is too low for an accurate AMS analysis of ^{14}C. One option would then either be to pool samples from two or more cases, and another possibility could be to run the isolated DG sample together with neuronal nuclei from the occipital cortex, which consistently have shown approximately the same age as the individual. Finally, it would be interesting to compare alcoholic subjects with and without alcohol dementia in order to better understand how this condition might be related to interference with DG neurogenesis, and loss of neurons in both DG and CA regions.

ACKNOWLEDGEMENTS

In the most excited journey of my life, I would like to express my gratitude to all the people who believed and supported me on my passion and dream towards science to come true.

I like to thank all the **donors** for donating their tissue samples for our research work. This thesis work is not possible without their contribution.

Prof. Henrik Druid, my main supervisor, for giving me an opportunity to work on this exciting research topic. Thank you for encouragement, for your motivation and belief on me, in scintillating my thinking and providing me an open learning environment. I never forget the call that I received from you in offering a PhD position at your lab during my visit to Denmark. Your enthusiasm, supervision and support have been indispensable for this thesis.

Prof. Nenad Bogdanovic, my co-supervisor, for introducing me to the world of stereology and your support has been invaluable throughout my PhD career. I admire your personal care for my development and I thoroughly enjoyed all our discussions.

Prof. Jonas Frisén, my mentor, for accepting my request to be a mentor to my PhD study and for inspiring us with your simplicity and thoughts.

I would like to thank my co-supervisor and my colleague, **Kanar Alkass**, for her valuable support in handling things and advice throughout the thesis. I like to thank **Prof. Deborah C. Mash**, for being a co-supervisor and providing samples to my study. I would like to thank **Tara Wardi Le Maître**, my colleague, friend and well-wisher for being there for me during difficult times and supporting me throughout the thesis. I thoroughly enjoyed our scientific discussions and also for sharing our views on different aspects of life. I would also like to thank **Charlotte Ovesen**, for helping me with administration stuffs during my beginning days as a PhD student. **Prof. Jens Randel Nyengaard**, for his constant support and guidance in understanding and working with stereological principles. I admire your scientific expertise and appreciate the pedagogic discussions that we have during the study period. I like to thank **Samuel Bernard** for the mathematical modeling and discussions. People at CMB: **Marcelo Toro** and **Sarantis Giatrellis**, thanks for helping me with nuclei sorting.

Friends at Stockholm: You guys made my life wonderful here at Stockholm. I like to thank **Navani** anna and **Jayanthi** akka for always being a moral support and for showing love and care on me. **Satish** anna, **Kani** akka, **Vijay** and **Ramya** for your love and support, thanks a lot for being my well-wisher. I also like to thank the little ones, **Nilan**, **Milir**, **Jugan**, **Krish** and **Kriwin** for making my time and life so light! **Pratheepan**, my friend and roommate, thanks a lot for your care and treating me like your brother always. You all are my family here at Stockholm. Thanks, **Ekambar** and **Lakshmi**, for all the fun filled conversations! Also, I would like to thank **Sakthi & Sunitha**.

Dear Family!

I feel truly blessed to have a wonderful family. My amma (**Jeevarani**) and appa (**Dhanabalan**), for their love and sacrifice in their life to uplift their children. My brother (**Dineshkumar**) and sister (**Sabari Sree**) for their love, care and of course fights altogether made our life so colorful. I thank my Bava (**Vidhyasagar**) for being a wonderful addition to our family. I would like to thank my Mamiya (**Muralidharan**) and Athamma (**Vijayalakshmi**) for adding me to their beautiful family. I also like to thank my Sister-in-laws, **Mohanapriya** and **Pavithra** for their love and care.

I would like to thank my Bava (**Thayumanaswamy**), Akka (**Vasantha Gowri**), Othina (**Kothai**), Athamma (**Jayalakshmi**) and Mamiya (**Janardhanan**) for helping me to make my first leap from Tamil Nadu to Stockholm.

Last but not least, my wife (**Radha Nandhini**) for showing unconditional love on me, for caring me as my mom and for supporting and encouraging me on all my endeavors. I am really fortunate to have you in my life.

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